

2012

IgE Enhances B Cell-Derived Exosomal Induced T Cell Proliferation

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IgE Enhances B Cell-Derived Exosomal Induced T Cell Proliferation

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
at Virginia Commonwealth University

By

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ACKNOWLEDGEMENTS

I would like to thank my graduate advisor and mentor, Dr. Daniel H. Conrad. I particularly appreciate that he took a chance on a non-traditional student.

I also thank my graduate committee members, Dr. Jessica Bell and Dr. Masoud Manjili for their support and guidance in and out of the classroom.

I would also like to thank Dr. Joel Mathews for giving me my start in the Conrad Lab and Dr. Jamie Sturgill for answering many questions.

I would also like to thank all of the current members of the laboratory for all of their support. Becca Martin has been especially patient and helpful with experiments, particularly with mouse work and flow cytometry. I would also like to thank former lab member Dae-Joong Kang who demonstrated a number of experimental procedures. I also thank Dr. Sarah Norton, Sheinei Saleem, Jennifer Bradley, and Lee Dean for putting up with me.

Finally, I must express my gratitude to Dr. Louis DeFelice, my interim advisor, who was instrumental in allowing me to pursue an advanced degree at Virginia Commonwealth University.

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LIST OF ABBREVIATIONS

ADAM	A disintegrin and metalloprotease
AHR	Airway hyperreactivity
Balb/c	Inbred mouse strain
BALF	Bronchoalveolar lavage fluid
C57BL/6	Inbred mouse strain
Ca ²⁺	Calcium ion
CD	Clusters of differentiation
DNP	2,4-Dinitrophenol
DO11.10	Inbred Balb/c mouse strain, expressing OVA-specific TCR
FBS	Fetal bovine serum
HeBS	HEPES Buffered Saline
Ig	Immunoglobulin
IgE	Immunoglobulin E
IL-4	Interleukin-4
i.n.	Intranasal
i.p.	Intraperitoneal
kD	Kilo Dalton
<i>Iz-CD23</i>	CD23 composed of a leucine zipper attached to portions of the extracellular domains of CD23
KO	Knockout

MHC	Major Histocompatibility Complex
miRNA	Micro ribonucleic acid
MVB	Multi vesicular body
OII	Inbred C57BL/6 mouse, expressing OVA-specific TCR
OVA	Ovalbumin
PBS	Phosphate Buffered Saline
RNA	Ribonucleic acid
TCR	T cell receptor
T _H 1	T helper cell type 1
T _H 2	T helper cell type 2
TNP	2,4,6-trinitrophenol, picric acid
WT	Wild-type

ABSTRACT

For many years it has been known that the injection of antigen bound to an antibody leads to more than a 1000-fold increase in antigen specific antibody response. This observation holds true for IgE, which is dependent upon CD23 expression, as this enhancement is not present in mice deficient in CD23. It also has been shown that when mice are injected with IgE-antigen complexes also display an increase in antigen specific T cell proliferation.

While there are published studies that demonstrate a role for B cell derived exosomes in the activation and proliferation of T cells, none have focused upon the potential role of CD23 as a molecular basis for this phenomenon, at least in the context of allergy and asthma. This thesis provides direct evidence that B cell-derived exosomes possess co-stimulatory molecules, including CD80 and CD86, which act in concert with CD23 to induce T cell proliferation, at least *in vitro*. This is due to, or enhanced by, the exosomal transfer of the antigen or peptide to T cells. Importantly, the antigen transfer is dependent upon the availability of IgE and the expression of CD23.

I. IgE and Allergy

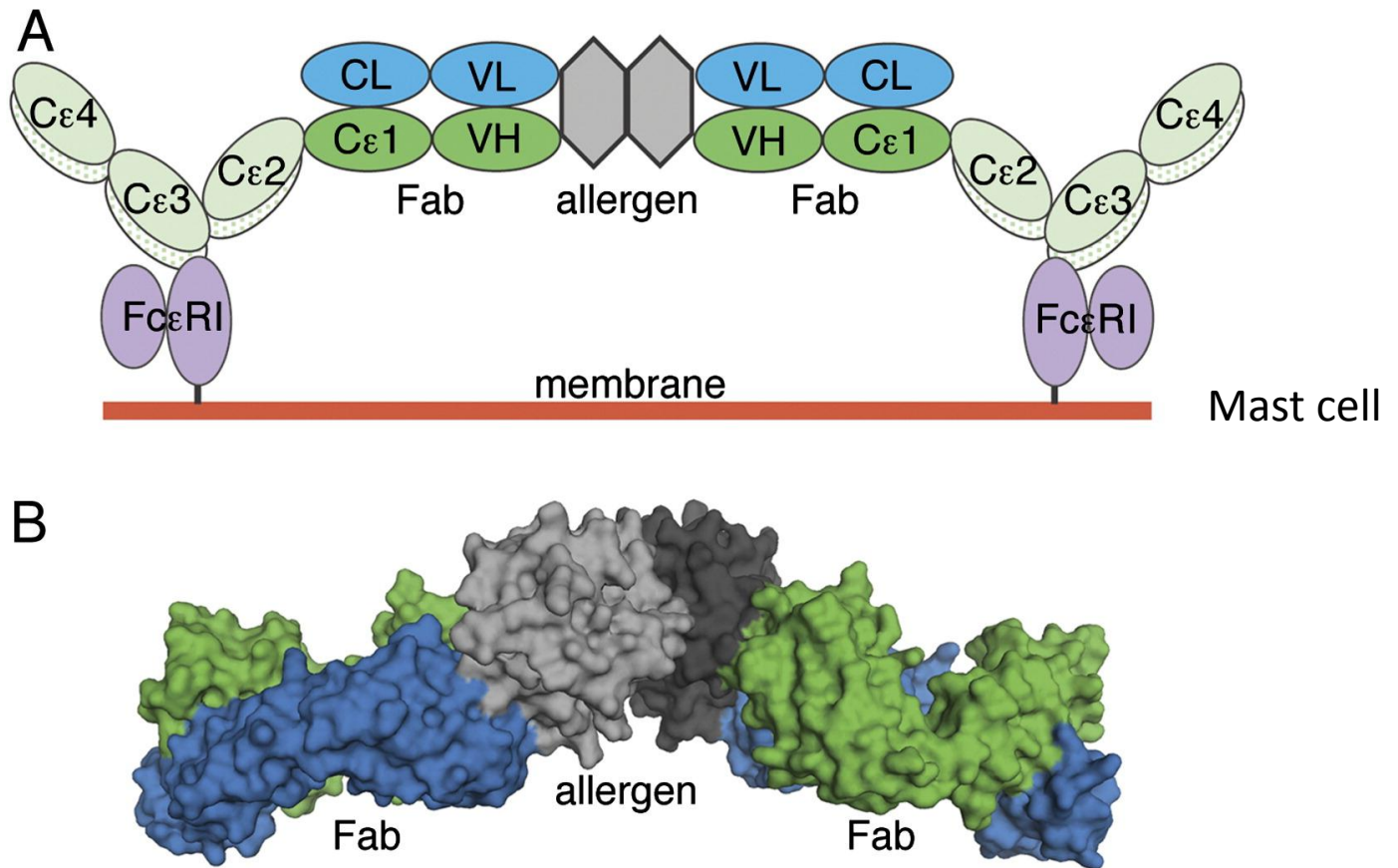
Type I hypersensitivity (allergy) is caused by the development and release of antibodies by activated B cells against what normally would be considered harmless proteins (allergens). Although most allergens are proteins, some are lipids, carbohydrates or proteases (*e.g.*, the dust mite allergen, Der p1).¹ Type I allergy reactions have emerged as one of the most common chronic diseases in the industrialized world, and is present across all age, sex, and ethnic groups.² Allergen exposure and sensitization elicits a T_H2-cell response in which Interleukin 4 (IL-4) and IL-13 drive an Immunoglobulin E (IgE) response due to class switch recombination in B cells.³ IgE, which is thought to have evolved as the immune system's main defense against helminths and parasitic pathogens, is the key mediator of allergic disease.⁴ The symptoms of Type I hypersensitivity arise when an allergen molecule binds to two IgE antibodies, which are bound to the high-affinity receptor for IgE, FcεRI, on the surface of mast cells or basophils.⁵ (See Figure 1) Cross-linking of adjacent IgE molecules by allergens exacerbates the allergic response, causing the release of biologically active products, such as histamine, lipid mediators, and newly produced cytokines leading to inflammatory reactions.⁶

IgE, as is true for other classes of antibodies, contains two identical heavy chains and two identical light chains. Whereas IgG contains two heavy chain domains in its Fc region, IgE contains three domains. (See Figure 2) Structurally, IgE is asymmetrically bent in the Fc region, with one Cε3 domain “open” and the other “closed” against the adjacent Cε2 domain.⁷ This structural asymmetry is a key feature of IgE's interaction with FcεRI.⁸ FcεRI is expressed on a variety of cells including: (1) on mast cells and basophils as a αβγ₂ tetramer; and (2) as an αγ₂ trimer on human (not murine) antigen presenting cells, monocytes, eosinophils, platelets, and

smooth muscle cells.⁹ (See Figure 3) IgE binding occurs on the extracellular domains of the *alpha* chain and signaling occurs via the immunoreceptor tyrosine-based activation motifs (ITAMs) located on the *beta* and *gamma* chains resident in the cytoplasm.

IgE also acts through FcεRII (known as CD23, the “low affinity” receptor)¹⁰. CD23 is distinguished from most other immunoglobulin receptors in that it belongs to the calcium-dependent (C-type) lectin family.¹¹ Interestingly, the name “low affinity receptor” is misleading; while the single head affinity for IgE is low, the naturally-occurring trimer exhibits affinity for IgE in the range of FcεRI. In the membrane bound form, CD23 is a trimer with three lectin domain “heads” tethered above the outer cell membrane by a triple α-helical coiled-coil stalk. (See Figure 4). IgE binding occurs in the head region of CD23. Proteolysis of the stalk region leads to the release of various forms of soluble CD23 (sCD23) whose biological activities depend on their oligomeric state.¹² Membrane-bound CD23 principally is cleaved by a disintegrin and metalloproteinase 10 (ADAM10) to form sCD23.¹³ CD23 exists in two forms, which differ in the N-terminal intracellular amino acid sequence – CD23a (first seven amino acids) and CD23b (first six amino acids). CD23a is expressed by activated B cells prior to differentiating into plasma cells; CD23b is expressed on inflammatory cells, epithelial cells, and B cells when exposed to IL-4. Unlike other lectins, CD23 does not depend upon carbohydrates to bind IgE;¹⁴ calcium ions (Ca²⁺), however, are required for IgE binding to CD23.^{15,16} While the crystal structure of CD23 is not completely known, it does appear that a CD23 trimer can bind three molecules of IgE, suggesting an expansive array of cross-linking on the cell membrane.¹⁷

Figure 1



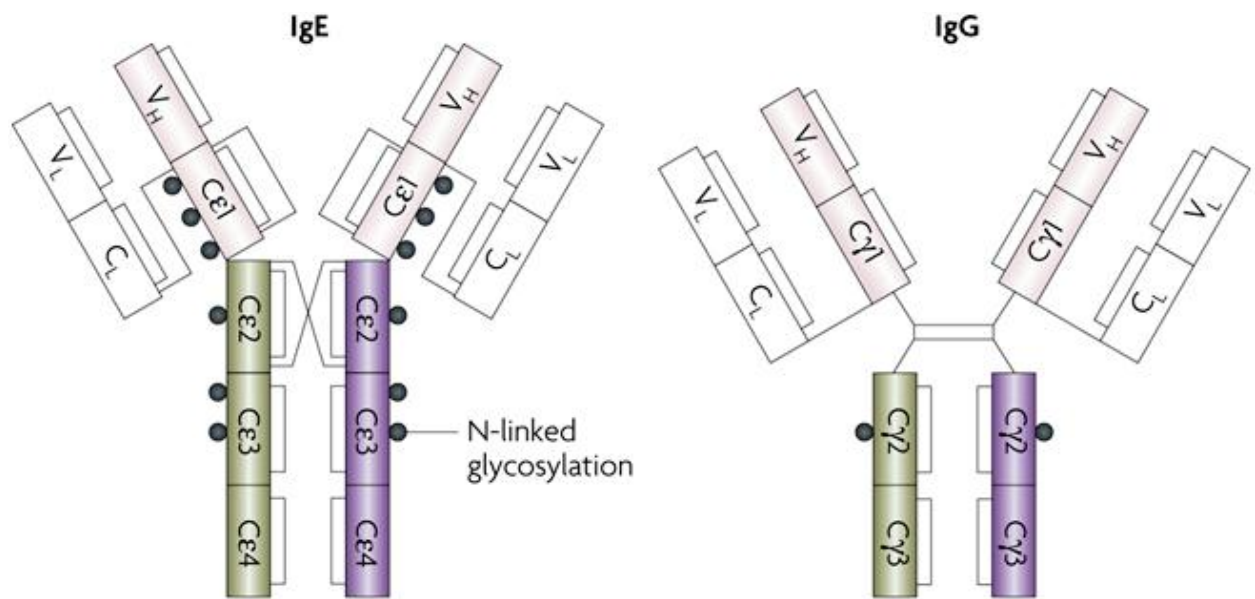
IgE Binds Allergens

A. Depiction of the binding of an allergen dimer (gray) by two IgE molecules bound to the Fc ϵ RI receptors on the surface of a mast cell.

B, The complex structure containing a β -lactoglobulin dimer in a complex with two Fab fragments.

Niemi, M., Jylhä, S., Laukkanen, M.L., Söderlund, H., Mäkinen-Kiljunen, S., Kallio, J.M., Hakulinen, N., Haahtela, T., Takkinen, K., Rouvinen, J., Molecular interactions between a recombinant IgE antibody and the beta-lactoglobulin allergen, *Structure*, 15(11):1413-21 (2007).

Figure 2



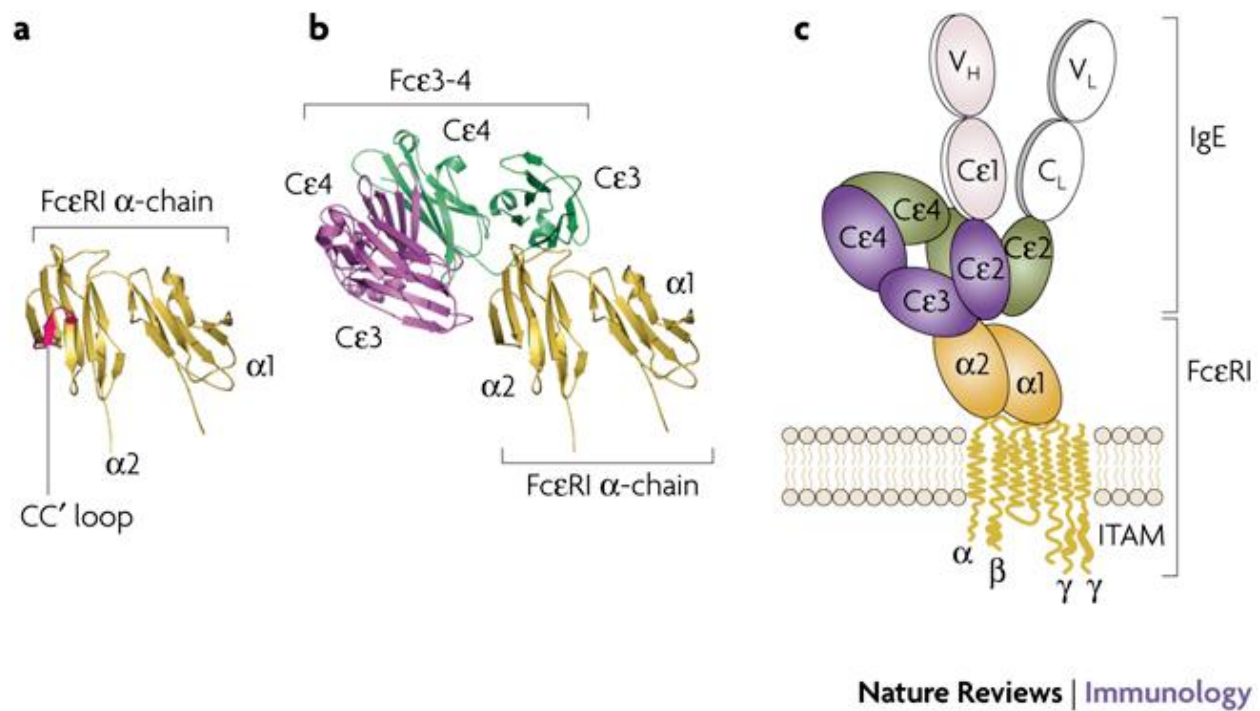
Nature Reviews | Immunology

Structure of IgE and IgG

IgE shares the same basic molecular structure as antibodies of other classes, with two identical heavy chains and two identical light chains. The heavy ϵ -chain contains one more domain than the heavy γ -chain of IgG. The pair of C ϵ 3 and C ϵ 4 domains are homologous in sequence, and similar in quaternary structure, to the pair of C γ 2 and C γ 3 domains of IgG. The pair of C ϵ 2 domains is the distinguishing feature of IgE.

Gould, H. and Sutton, B.J. IgE in allergy and asthma today. *Nature Review Immunology*. 8, 205-217 (2008).

Figure 3

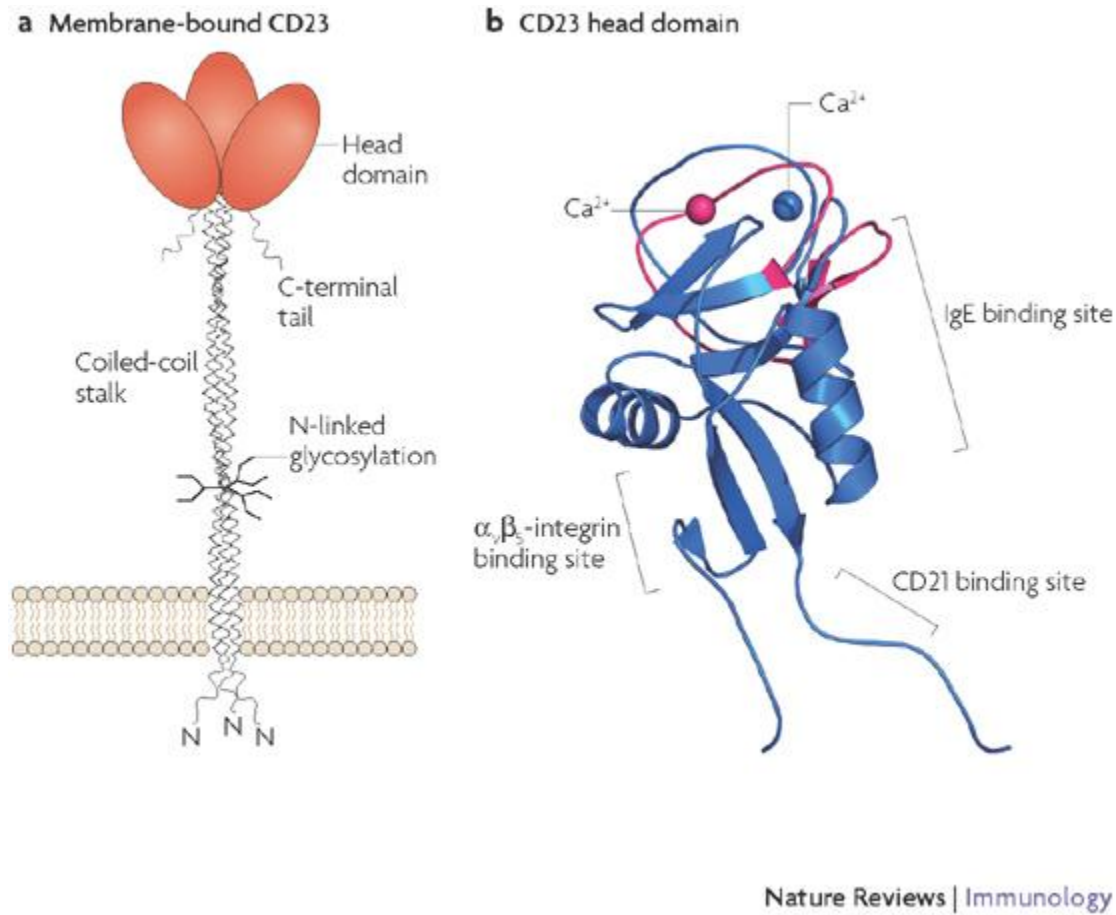


Structure of FcεRI

The structure of the extracellular domains of the FcεRI α-chain taken from the crystal structure of the FcεRI α-chain complex. Also, a representation of the entire IgE molecule bound to the extracellular domains of the FcεRI α-chain, with a depiction of the immunoreceptor tyrosine-based activation motifs (ITAMs).

Gould, H. and Sutton, B.J. IgE in allergy and asthma today. *Nature Review Immunology*. 8, 205-217 (2008).

Figure 4



Structure of FcεRII (CD23)

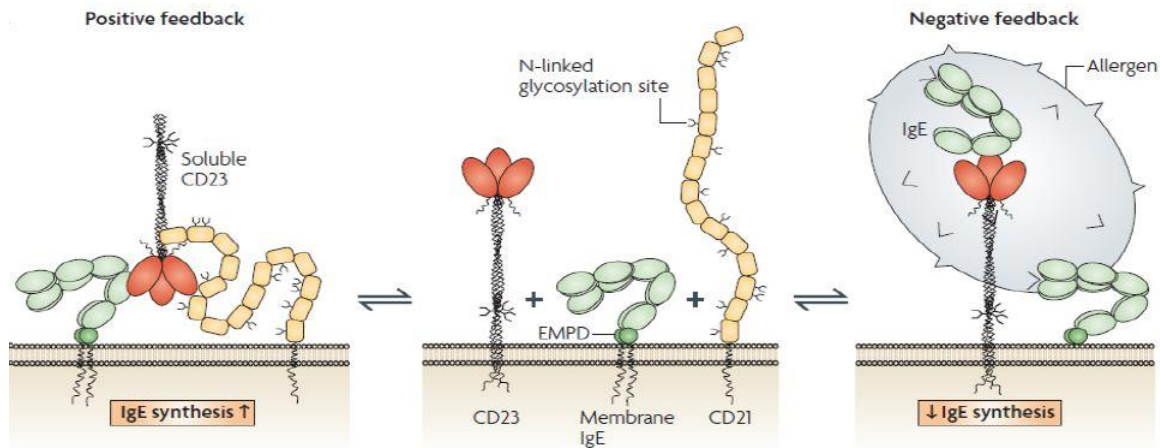
An illustration of membrane-bound CD23, showing the extracellular trimeric α -helical coiled-coil stalk region, three C-type lectin domain heads and the C-terminal tails. Also indicated are the glycosylation sites near the base of the stalk.

Gould, H. and Sutton, B.J. IgE in allergy and asthma today. *Nature Review Immunology*. 8, 205-217 (2008).

CD23 provides several regulatory functions which impact the effects of high levels of IgE in allergic disease. IL-4 and IL-13 induce class switch recombination for IgE synthesis and also induce B cells and inflammatory cells to up regulate expression of CD23. As IgE levels increase, CD23 binds to free IgE molecules, thereby acting as a buffer by blocking antibody access to available FcεRI receptors. Additionally, it is believed that CD23 can stimulate and repress IgE expression through positive and negative feedback loops.¹⁸ In humans, but not mice, one hypothesis proposes the existence of a positive feedback loop in which ADAM10 cleaves membrane bound CD23, allowing sCD23 to co-ligate membrane IgE and CD21^{fn1}.¹⁹ These three molecules then form an extensive array and act to induce B cell proliferation and differentiation into IgE-producing plasma cells. IgE is believed to be down regulated (negative feedback loop) when cleavage of membrane bound CD23 is blocked so that sCD23 is prevented from forming the CD21/membrane IgE signaling platform.²⁰ The existence of a positive feedback loop provides a therapeutic target: if proteolysis of CD23 can be blocked, IgE levels can be kept at levels that do not cause allergic disease. (See Figure 5)

¹ CD21 is called also B2 and is identical with the complement fragment C3d receptor known as CR2 (complement receptor-2). CD21 is the major receptor for C3d fragments on immune complexes.

Figure 5



Positive and negative regulation of IgE synthesis by human CD23. In this model, positive regulation of IgE synthesis is a result of the co-ligation of membrane IgE and CD21 on a human B cell committed to IgE synthesis by soluble CD23 released from membrane-bound CD23. The negative signaling pathway of CD23 is unknown.

Gould, H. and Sutton, B.J. IgE in allergy and asthma today. *Nature Review Immunology*. 8, 205-217 (2008).

II. Exosomes

Membrane proteins may be internalized through clathrin-coated pits and delivered to early endosomes. In the early endosomes, internalized molecules are either recycled to the plasma membrane or sequestered in internal vesicles. Multivesicular endosomes, also known as multivesicular bodies (MVBs), are intermediates in the degradation of proteins internalized from the cell surface or sorted from the trans Golgi network.²¹ MVBs may fuse with lysosomes where the contents are degraded through hydrolase enzymatic action. In a separate pathway, MVBs fuse with the plasma membrane and internal vesicles within the MVB are released into the extracellular space as exosomes.²² (See Figure 6) Exosomes display the same orientation as the plasma membrane, with extracellular domains of proteins on the surface.

Exosomes were first reported in the early 1980s by Trams, Lauter, *et al.*, in connection with research on phosphoesterhydrolases from C-6 glioma or N-18 neuroblastoma microvesicles, who remarked that exfoliated membrane vesicles may serve a physiologic function; the authors proposed that the vesicles be referred to as exosomes.²³ These microvesicles were further characterized by two different research groups studying the recycling of the transferrin receptor in reticulocytes.^{24,25} (See Figure 7 and Figure 8). After these reports, exosome research became fairly dormant.

Renewed interest in microvesicle research was stimulated in 1996 when Raposo reported that Epstein–Barr virus -transformed B-lymphocytes secreted exosomes that bore major histocompatibility class (MHC) II dimers bound to antigenic peptides, a complex essential for the adaptive immune response.²⁶ Subsequently, Raposo demonstrated that dendritic cells (the immune cells that initiate adaptive immune responses by presenting MHC–peptide complexes to

naïve T cells) also secrete exosomes bearing functional MHC–peptide complexes, which could promote induction of antitumor immune responses in mice *in vivo*. These results provided the basis for the hypothesis that exosomes could play an active role in intercellular communication, at least in the immune system.²⁷

Exosomes originate in the late endosomal compartment and constitute a homogenous population with a size ranging from as small as 30 nm with an upper range of 120 nm (the limits of the range is the subject of dispute).²⁸ Exosomes have been isolated from *in vitro* cultures of virtually every cell type (from mammals, birds, fish, parasites and plants), including intestinal epithelial cells, Schwann cells, and tumor cell lines, but also from primary immune system cells such as reticulocytes, mast cells, dendritic cells (DCs), platelets, B lymphocytes and T lymphocytes. In addition, exosomes have been purified from several body fluids, including serum and plasma, human bronchoalveolar fluid (BALF), urine, milk or colostrum, and bile.

Exosomes can be isolated through several means,²⁹ including multi-step differential centrifugation (probably the most commonly utilized method), density gradient purification, immunoaffinity capture, and a proprietary precipitation method called ExoQuickTM.³⁰ In general, to qualify as an exosome, the vesicle must present the following biophysical properties:³¹

- (a) limited by a bilipidic layer;
- (b) small diameter (30 to 120 nm);
- (c) a cup-shaped morphology in electron microscopy; and

(d) floatation on a sucrose gradient with their density between 1.10 - 1.21 mg/mL, depending on the producing cell type. (See Figure 9)

The isolation of exosomes can be further validated by the presence of canonical surface markers, including HSP 70, CD9, MHC class I, MHC class II, and/or CD63.³² (See Figure 10)

Although their exact role in is not known, exosomes have been shown to influence many biological processes and diseases. For example, exosomes contain signal proteins, peptides, miRNAs, mRNAs, and lipids. Exosomes are known to act as messengers between cells by eliciting cytokines, and by transferring miRNA, proteins, and antigens to other cells.³³ In fact, one particularly interesting study showed that miRNAs are exchanged during cognate immune interactions, demonstrating the existence of antigen-driven unidirectional transfer of miRNAs from the T cell to the APC, mediated by the delivery of CD63⁺ exosomes on immune synapse formation.³⁴ The study further demonstrated that inhibition of exosome production through sphingomyelinase-2 impaired the transfer of miRNAs to APCs. Similarly, it has been hypothesized that one cell communicates with other cells by releasing RNA-containing exosomes and modulating the target cell's protein production.³⁵

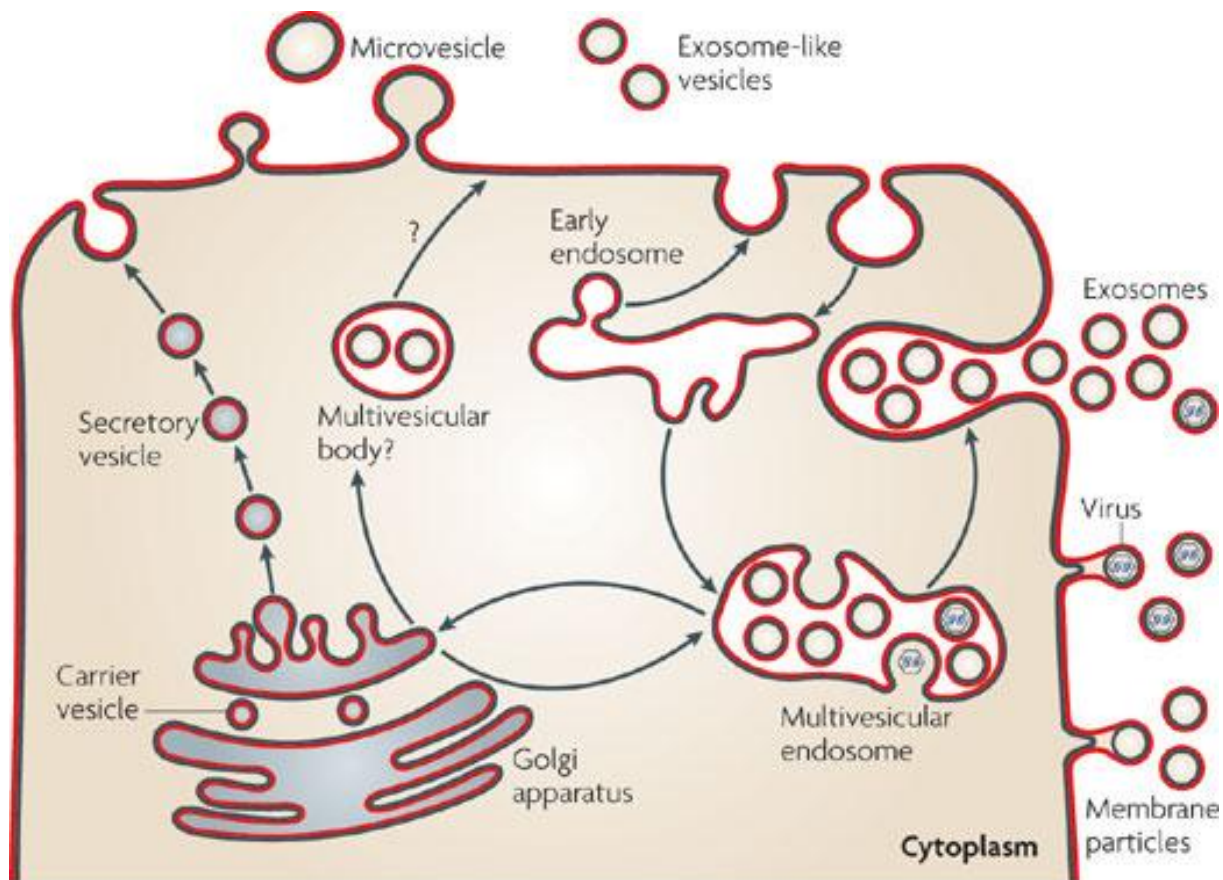
Tumors of the breast, oral cavity, colorectal, brain, ovary, bladder, prostate, and melanomas have been reported to release exosomes.³⁶ In cancer, exosomes can play a positive and negative role. While exosomes aid in the removal of potentially detrimental or unneeded molecules from cells, tumor-derived exosomes can remove therapeutic drugs. Moreover, cells that have been exposed to chemotherapy release drug-containing exosomes, indicating a resistance to treatment.³⁷ On the other hand, dendritic cells co-cultured with peptide derived from a variety of different tumors mediate a reduction in tumor growth in an MHC and CD8+

T cell-dependent manner.³⁸ Due to this ability to present antigen to immune effector cells, dendritic cell exosomes present a possible treatment for cancer.

III. Exosomes and Allergy

Exogenous antigens (inhaled, ingested, or injected) are taken up by antigen-presenting cells (APCs) such as phagocytic cells including dendritic cells and macrophages. In brief, APCs capture an antigen by endocytosis, whereby the cell engulfs some of its extracellular fluid and any material dissolved or suspended in that boundary. That portion of the plasma membrane is invaginated and forms a membrane-bounded vesicle called an endosome. The endosome then fuses with a lysosome where the antigen is degraded into short peptides, which in turn displayed at the surface of the cell within a MHC Class II molecule.³⁹ (See Figure 11) Once the peptides are displayed, they may be recognized by the T cell receptors (TCRs) present on CD4⁺ T cells (a process known as “priming”). In turn, the newly conferred antigen-specific T cells recognize the antigens presented by B cells and stimulate the B cells through CD40 ligand (anti-CD40) interactions with CD40. The activated B cells then up regulate costimulatory molecules such as CD80 and CD86 that are then able to further stimulate T cells to produce cytokines inducing B cells to switch isotypes and differentiate to produce antibodies, such as IgE.⁴⁰ (See Figure 12)

Figure 6



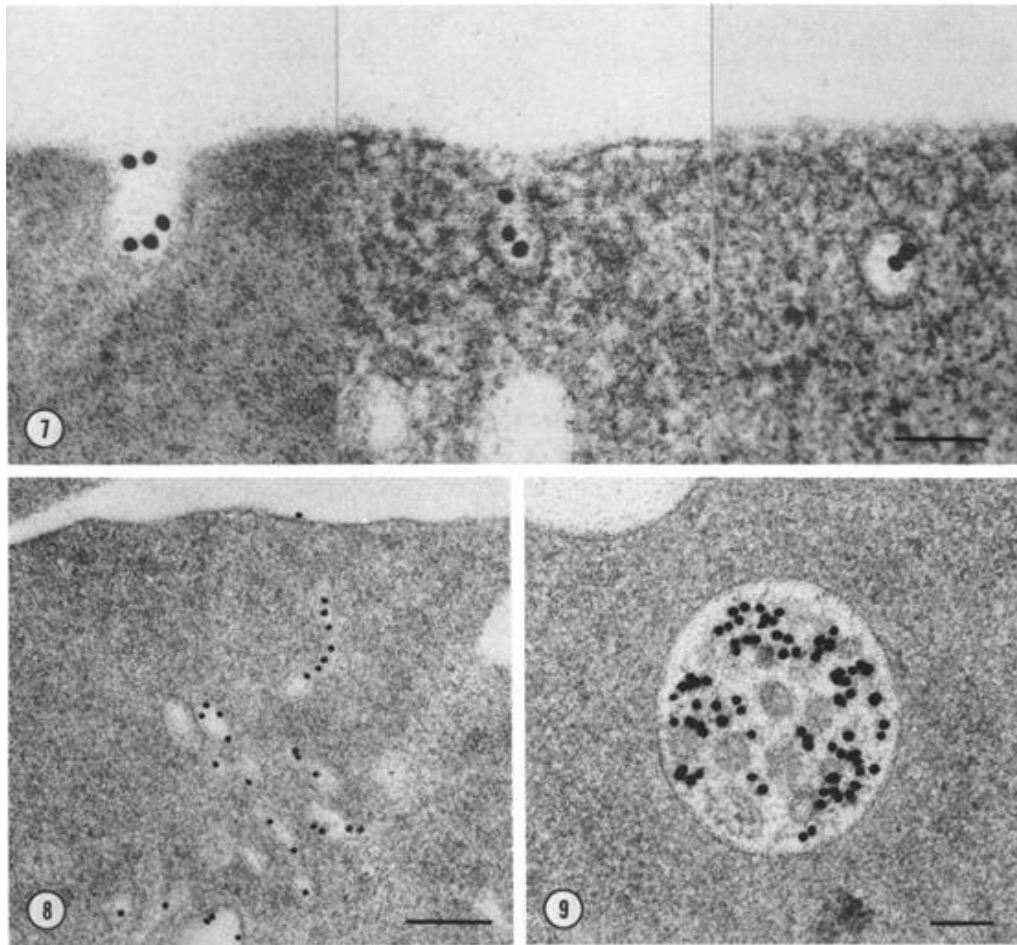
Nature Reviews | Immunology

Intracellular Pathways of Endosomes

Intracellular trafficking occurs through carrier and secretory vesicles that contain intraluminal components. By contrast, secreted membrane vesicles contain cytoplasmic constituents. Secreted membrane vesicles can form at the plasma membrane by direct budding into the extracellular space, giving rise to microvesicles and membrane particles. Secreted vesicles also can form inside internal compartments from where they are secreted by fusion with the plasma membrane.

Théry, C., Ostrowski, M., and Segura, E., Membrane vesicles as conveyors of immune responses, *Nature Reviews Immunology*, 9, 581-593 (2009)

Figure 7

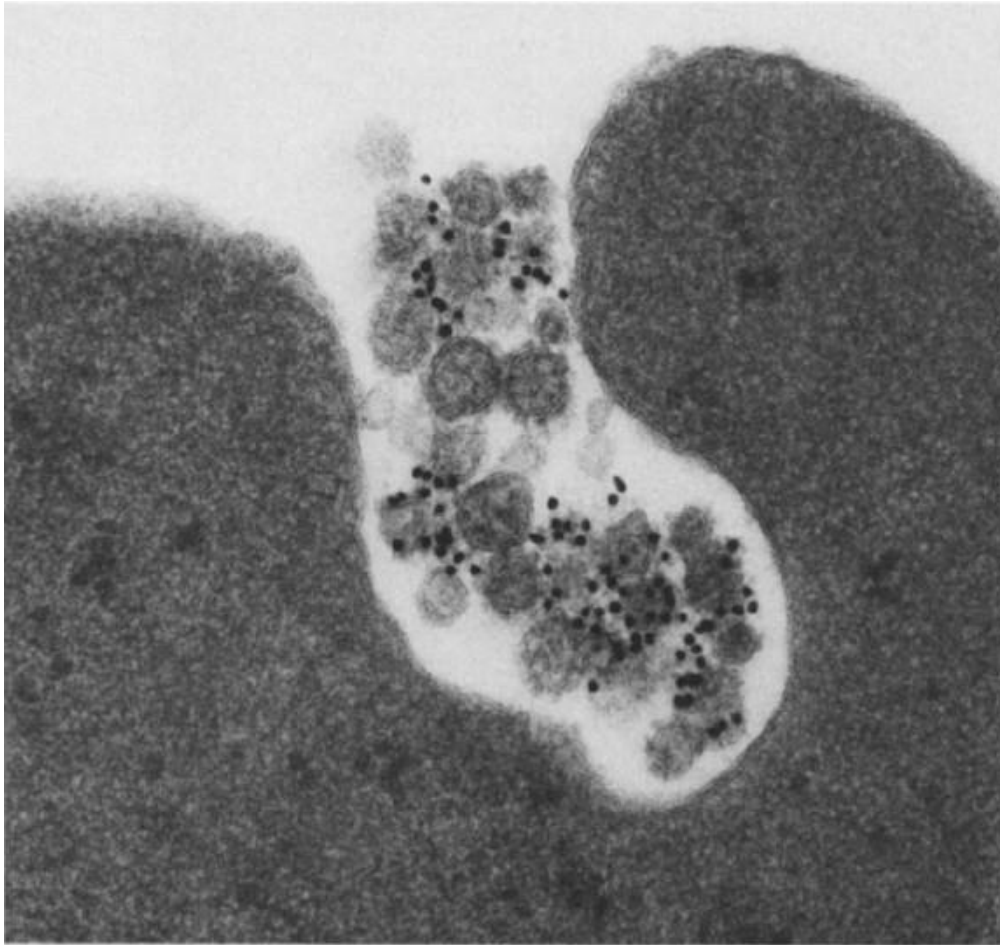


Images of Exosomal Release by Electron Microscopy

Thin sections of reticulocytes incubated with colloidal gold-transferrin (AuTf) for 5 min at 37°C. AuTf is localized to the free surface of the plasma membrane, within coated pits and vesicles (~100 nm o. d.), inside small uncoated vesicles of varying size (<200 nm; generally 100 nm diameter), and inside tubular structures (usually 60-80 nm diameter). Some vesicular profiles may represent cross sections of tubular compartments. The clathrin coat is sometimes difficult to discern in thin sections, since the reticulocyte cytoplasm stains so densely that it can mask the presence of a densely staining coat on the cytoplasmic surface of membrane structures. When reticulocytes were incubated for 20 min or longer at 37° C, AuTf was also found within MVE.

Harding, Heuser, and Stahl. Receptor-mediated Endocytosis of Transferrin and of the Transferrin Receptor in Rat Reticulocytes. *The Journal of Cell Biology*, 97, 329-339 (1983).

Figure 8



Close-up of Exosomal Release

Fusion of multi vesicular bodies (MVB) with the plasma membrane and release of round bodies. The small dense bodies are labeled with gold and is only present on the 50-nm bodies which are inside vesicles of 300-800 nm in diameter. The limiting membrane of the MVB does not contain the gold label.

Johnstone, R., Electron Microscopic Evidence for Externalization of the Transferrin Receptor in Vesicular Form in Sheep Reticulocytes, *The Journal of Cell Biology*, 101, 942-948 (1985).

Figure 9

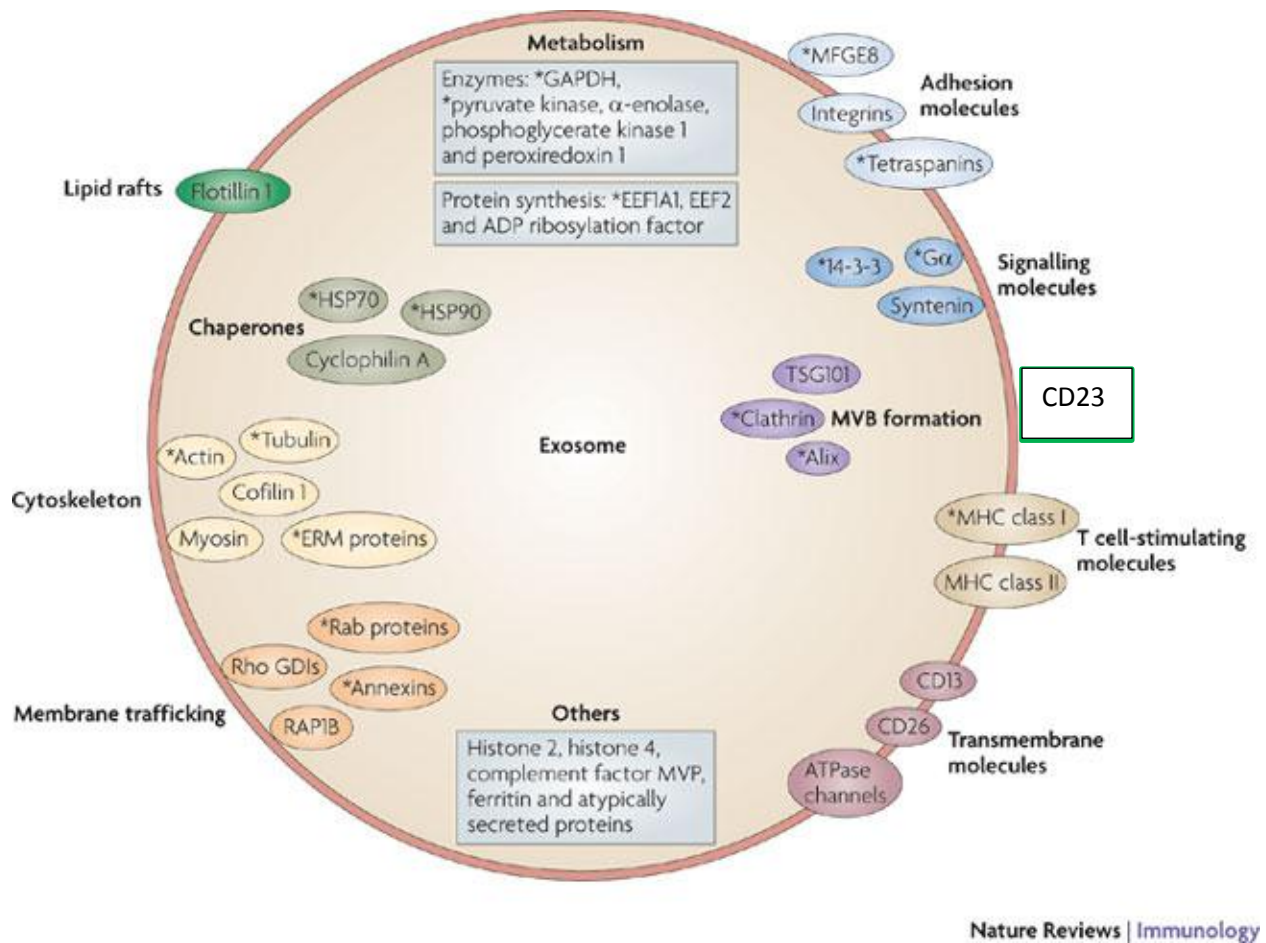
Table 1 Physicochemical characteristics of different types of secreted vesicle						
Feature*	Exosomes	Microvesicles	Ectosomes	Membrane particles	Exosome-like vesicles	Apoptotic vesicles
Size	50–100 nm	100–1,000 nm	50–200 nm	50–80 nm	20–50 nm	50–500 nm
Density in sucrose	1.13–1.19 g/ml	ND	ND	1.04–1.07 g/ml	1.1 g/ml	1.16–1.28 g/ml
Appearance by electron microscopy†	Cup shape	Irregular shape and electron-dense	Bilamellar round structures	Round	Irregular shape	Heterogeneous
Sedimentation	100,000 g	10,000 g	160,000–200,000 g	100,000–200,000 g	175,000 g	1,200g, 10,000 g or 100,000 g
Lipid composition	Enriched in cholesterol, sphingomyelin and ceramide; contain lipid rafts; expose phosphatidylserine	Expose phosphatidylserine	Enriched in cholesterol and diacylglycerol; expose phosphatidylserine	ND	Do not contain lipid rafts	ND
Main protein markers	Tetraspanins (CD63, CD9), Alix and TSG101	Integrins, selectins and CD40 ligand	CR1 and proteolytic enzymes; no CD63	CD133; no CD63	TNFR1	Histones
Intracellular origin	Internal compartments (endosomes)	Plasma membrane	Plasma membrane	Plasma membrane	Internal compartments?	ND
Main reference	142	3	143	8	17	144

Characteristics of Secreted Vesicles

Secreted vesicles represent a heterogeneous class and are separated into types of vesicles based upon size and composition.

Théry, C., Ostrowski, M., and Segura, E., Membrane vesicles as conveyors of immune responses, *Nature Reviews Immunology*, 9, 581-593 (2009)

Figure 10



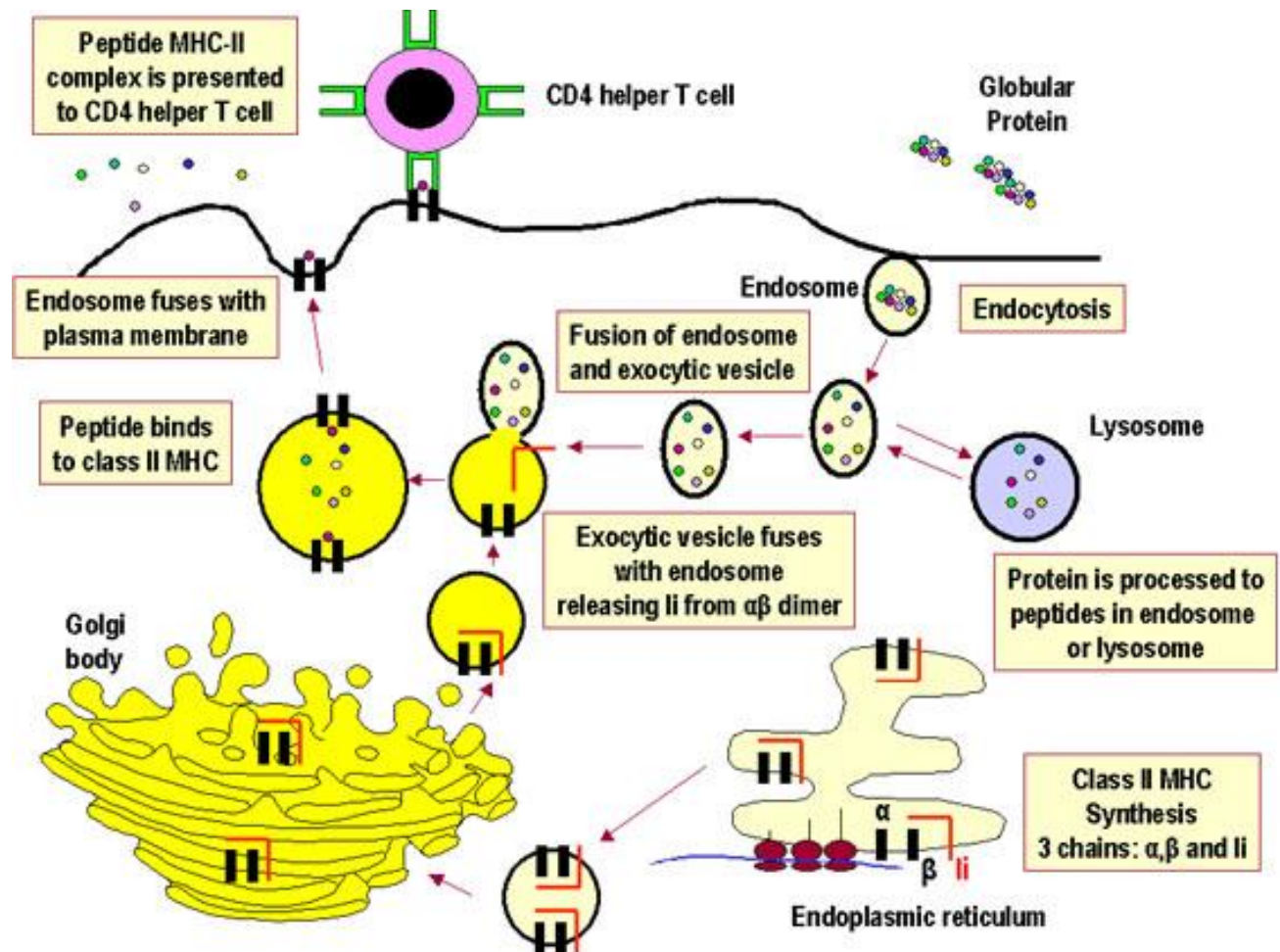
Exosomal Surface Markers

Proteins found in at least 30% of different exosomes are listed and proteins present in at least 50% of exosomes are indicated by an asterisk.

Théry, C., Ostrowski, M., and Segura, E., Membrane vesicles as conveyors of immune responses, *Nature Reviews Immunology*, 9, 581-593 (2009)

The activation of T lymphocytes is mediated by the interaction of TCRs with their ligands, major histocompatibility molecule-peptide complexes.⁴¹ The immune synapse, the interactions between APCs presenting antigen to T cells, is a critical step in the process of eliciting T_H2 cell proliferation and maturation, and inducing B cell isotype switching. (See Figure 13) Exosomes from APCs bear MHC class I and II and molecules including CD54, CD80, and CD86 known to co-stimulate T cells. The precise mechanisms underlying exosome-induced stimulation, however, have not been elucidated. One line of studies demonstrates that exosomes can stimulate T cells directly, whereas others indicate that exosomes must act in conjunction with other APCs.⁴² As noted previously, exosomes from APCs (especially dendritic cells and B cells) have been shown to carry antigens and antigen complexes.⁴³ In one notable study, exosomes isolated from B cells were shown to present allergen-derived peptides and to induce T-cell proliferation and the production of high levels of IL-4, IL-5, and IL-13 and lower levels of IFN- γ and TNF- α , a cytokine profile consistent with an allergen-induced response.⁴⁴ Similarly, B cell exosomes loaded with birch pollen peptide also have been shown to induce Bet v 1-specific T cell proliferation in a dose dependent manner.⁴⁵

Figure 11

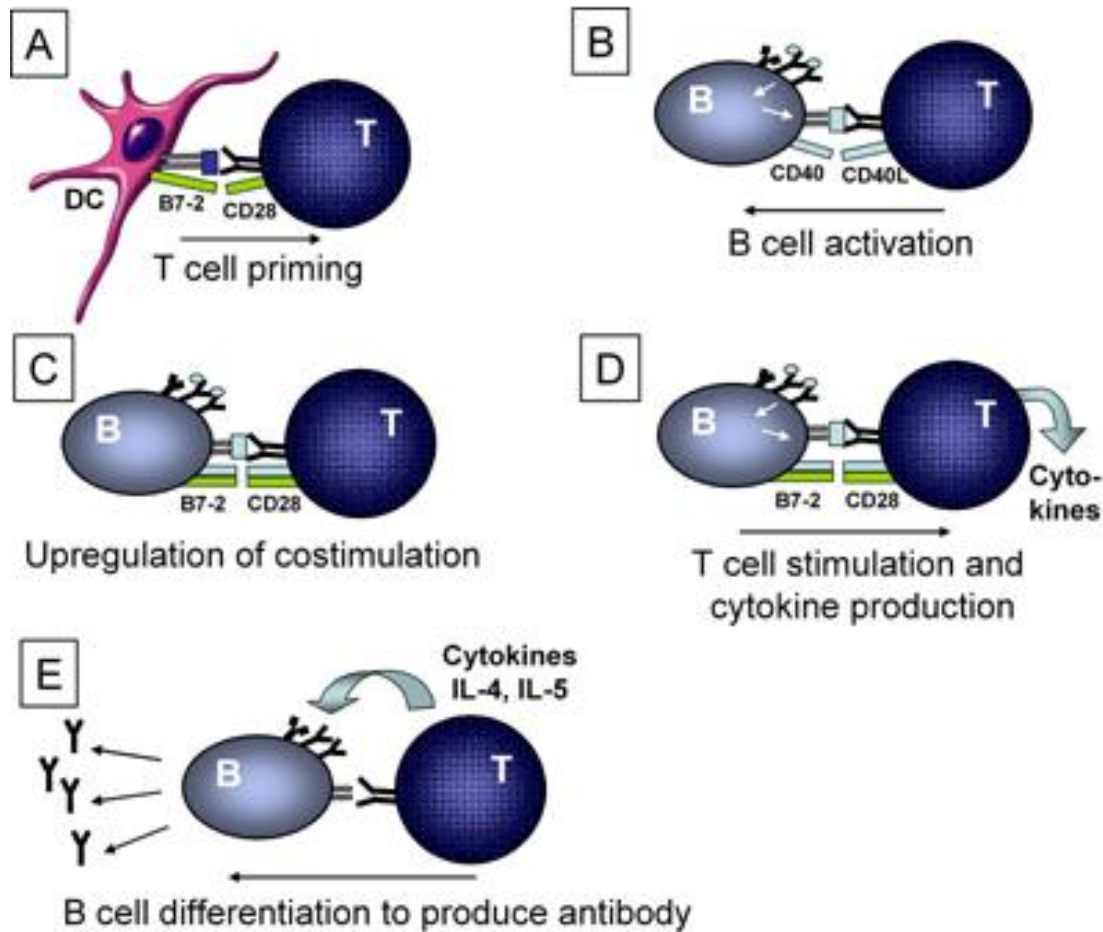


MCH Class II Pathway

Exogenous proteins taken in by endocytosis are fragmented by proteases in an endosome. The alpha and beta chains of MHC class II, along with an invariant chain, are synthesized, assembled in the endoplasmic reticulum, and transported through the Golgi and trans-Golgi apparatus to reach the endosome, where the invariant chain is digested, and the peptide fragments from the exogenous protein are able to associate with the class II MHC molecules, which are finally transported to the cell surface. The exogenous proteins are then displayed for antigen presentation to T helper cell.

<http://pathmicro.med.sc.edu/bowers/ant-pres.htm>

Figure 12

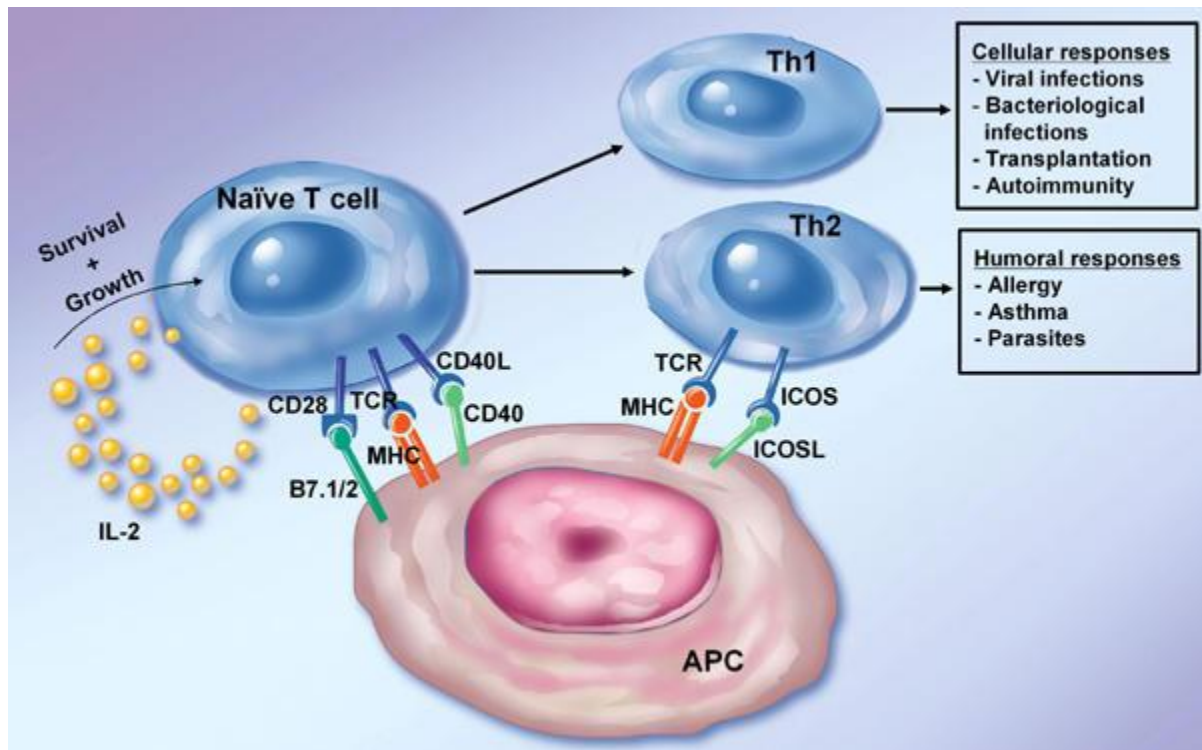


T Cell and B Cell Interactions and Antibody Production

Antigen-presenting cells prime naive T cells. Antigen-specific T cells recognizing antigens presented by B cells in turn stimulate the B cells through CD40/anti-CD40 interactions. Activated B cells up regulate costimulatory molecules such as CD80 and CD86 which are then able to stimulate T cells. T cell-derived cytokines stimulate B cells to switch isotypes and differentiate to produce antibodies.

Wong, F. S., Wen, L., *B Cells in Autoimmune Diabetes*, Review Diabetic Studies, 2(3):121-135 (2005).

Figure 13



The Immune Synapse

The immune synapse, the interactions between APCs presenting antigen to T cells, is a critical step in the process of eliciting T_H2 cell proliferation and maturation, and inducing B cell isotype switching.

Boehm, B. and Bluestone, J. Differential Roles of Costimulatory Signaling Pathways in Type 1 Diabetes Mellitus, *Rev. Diabet. Stud.* Winter; 1(4): 156–164 (2004)

IV. Experimental Procedures

Reagents

Rabbit anti-mouse --anti LZ CD23 (diluted 1:250/500), extracellular CD23 held in a trimer by the addition of a leucine zipper motif was created as previously described.⁴⁶ Rabbit anti-mouse CD9 (Sigma C9993) (diluted 1:500/1000). Mouse anti mouse MHCII H2-I/Ad β (5K43) antibody (Santa Cruz sc-71202) (diluted 1:500/1000). Rabbit anti-mouse Mouse IgE, Fc specific antibody (Acris AP21485AF-N, diluted 1:500/1000). Rabbit anti-OVA (Abcam ab1221), rabbit anti-CD80 antibody (ab64116) and Anti-CD86 antibody (ab112490) were purchased from Abcam, Cambridge, Massachusetts. Goat anti-rabbit HRP was purchased from Southern Biotech (Birmingham, Alabama). Mouse IL-4 was a gift from Bill Paul (National Institutes of Health).

Mice

BALB/C, DO11.10, OTII and C57BL/6J mice were purchased from Jackson Laboratory (Bar Harbor, Maine) and were used in connection with the generation of exosomes, AHR experiments, and in the T cell proliferation studies. Female and male mice ages 8-12 weeks were used in the experiments. All mouse protocols were approved by the Virginia Commonwealth University Institutional Animal Care and Use committee.

Cell Culture

Cells used in connection with the exosome studies described herein were cultured in Complete RPMI, containing 5 mL Penicillin/Streptomycin, 10 mL HEPES, 5mL non-essential amino acids, 5 mL sodium pyruvate, 5 mL L-glutamine, 500 μ L 2 mercaptoethanol, 50 mL fetal bovine serum, 200 μ L Gentamycin, and RPMI 1640 added *quantum sufficit* to 500 mL. The FBS

was centrifuged at 100,000 x g for 2-4 hours to remove possible bovine exosomes prior to preparation of the cRPMI.

Isolation of Primary Cells from Mouse Spleens

After removal, the spleen was gently crushed between sterile microscope slides, the cells were placed into 50mL sterile tubes and centrifuged at 1,500 rpm for 5 minutes. After pouring off the supernatant, 10 mL of ACK Lysis Buffer was added to the cell pellet, gently mixed and placed on ice. After 5 minutes the cells were and centrifuged at 1,500 rpm for 5 minutes. The cell pellet was resuspended for cell selection using Miltenyi Biotec MACS Cell Separation Microbeads and LS and/or LD columns according to manufacturer's instructions. CD45R (B220), Order No. 130-049-501, were used to isolate B cells; B cell purity of 98% or greater was established by flow cytometry. For DO11.10 T cell isolation, a cocktail of CD45R, CD11b (Order No. 130-049-601) and CD11c (Order No. 130-052-001) Microbeads was prepared and added to a single cell suspension of spleen cells. The labeled cells were passed through either an LS or LD column. The flow through cells were then labeled with L3T4 Microbeads (Order No. 130-049-201) and passed through an LS column. The labeled (captured) cells were then used for T cell proliferation studies. DO11.10 T cell purity of 95 % was confirmed by flow cytometry.

Isolation of Exosomes

Exosomes were isolated as previously described. Briefly, primary mouse B cells were plated in 6 well plates at a concentration of 1 million cells per milliliter and then cultured for 2 or 3 days with IL-4 and anti-CD40. The cells were pelleted by spinning them at 300 x g for 5 minutes, then 2,000 x g for 10 minutes and supernatants were harvested. To remove any possible

apoptotic bodies the supernatants were spun at $27,000 \times g$ (Sorvall RC 5B Superspeed Centrifuge, SS34 rotor) for 20 minutes. The supernatants were passed through a $0.2 \mu\text{m}$ filter to remove particles larger than 200 nm. Finally exosomes were harvested by spinning at $1 \times 100,000 \times g$ (Sorvall AH-650 or AH-629 rotor) for 1 hour; the exosome pellet was resuspended in 5 mL of PBS or HeBS and pelleted again at $100,000 \times g$ for 1 hour. When isolating exosomes for CD23 studies, the exosome pellet was resuspended in 100 μL or 200 μL of HeBS with Ca^{2+} , otherwise the pellet was resuspended in PBS. Electron microscopy was used on some samples to confirm that the isolated particles were exosomes. Bradford Assays or Nanodrop exosome yields were approximately 1.0-1.62 $\mu\text{g}/\mu\text{L}$, depending upon the length of time that B cells were in culture.

Western Blots

Western Blots were performed using the Life Technologies XCell SureLock Mini-Cell Electrophoresis System and 10% Bis-Tris gels for samples. NuPage MES SDS Running Buffer and NuPage MES Transfer Buffer were used according to manufacturer's instructions. Briefly, 20 μg of sample per lane was pipetted into the gel and the apparatus ran for 35 minutes at 200V, 120mA. Transfer of the samples on to nitrocellulose paper was accomplished using a BioRad for 1.5 hours at 100V, 200mA or overnight in a cold room at 25V.

Blocking was performed either in a 5% powdered milk solution (HeBS or PBS), plus 0.05% v/v Tween-20 for two hours on an oscillating platform. Generally, the primary antibody was allowed to bind in a cold room overnight.

For mouse CD23 Western blots, CD23 was detected with rabbit anti-mouse CD23 followed by goat anti-rabbit HRP antibodies. For MHC class II blots, the proteins were detected using rabbit anti-H2-I/Ad β followed by goat anti-rabbit HRP-conjugated secondary antibodies.

Separation of CD23⁺ and CD23⁻ Exosomes

(a) Bio-Rad Affi-Gel 10 Coupling

IgE was coupled to Bio-Rad Affi-Gel 10 according to manufacturer's instructions. In brief, 40 mL of cold isopropanol and then 40 mL of PBS was washed over 5mL of Affi-Gel on a Buchner funnel. Using a spatula, the Affi-Gel was placed into a 15 mL tube. Next, 10 mg of IgE (first dialyzed against PBS) was added to the Affi-Gel. 5 mL PBS was then added to create a 50% slurry, to which 100 μ L of 1M HEPES was added, to a pH of 7.4. After breaking up clumps with a spatula, the slurry was placed on a nutator in a cold room (4°C) overnight. The next day the slurry was washed 5 times in 5 mL PBS by centrifugation at 1,000 rpm, turning off machine once 1,000 rpm is reached. The first three supernatants were kept to assess protein content by Bradford Assay. The Affi-Gel slurry was then treated with 500 μ L 2M Tris (pH 8.0-8.2) + 4.5 mL PBS and placed on a nutator in a cold room (4°C) overnight. The next day the slurry was washed by centrifugation 3 times in 5 mL PBS and resuspended in 5mL PBS, plus 10 mg of NaN₃ and stored at 4°C.

(b) Affi-Gel Exosome Separation

To prepare Aff-Gel slurry, stock Affi-Gel was washed two times with 5 mL PBS, spun to 1,000 rpm and the centrifuge was turned off, then pouring off the supernatant. The Affi-Gel was resuspended in 5 mL PBS. Next, 1 mL of slurry was placed in a 5 mL Falcon tube. Once again, the slurry was spun to 1,000 rpm and the centrifuge was turned off, and the supernatant was poured off. Approximately 500 μ L of Affi-Gel remained in the tube. The exosome pellet from first 100,000 g spin, was resuspended in 1 mL HeBS plus Ca²⁺ and added to the 500 μ L IgE Affi slurry. The slurry was nutated in a cold room for 1 hour. After the slurry was spun to 1,000 rpm, the supernatant was pipetted, retaining both the supernatant and gel pellet. To gel

supernatant (CD23⁻ fraction), HeBS plus Ca²⁺ was added up to a total volume of 5 mL and placed in ultracentrifuge tube for second 100,000 g spin. To recover CD23⁺ exosomes, 4 mL HeBS without Ca²⁺ was added to exosome pellet and 500 µL of 10mM EDTA (1:50 dilution of .5M stock) was added to release the CD23⁺ exosomes from the Affi-Gel. The mixture was centrifuged to 1,000 rpm and supernatant was retained. An additional 500 µL of the EDTA solution was added to the pellet, and centrifuged to 1,000 rpm and once again retaining the supernatant. Both supernatants were added to a 5 mL ultracentrifuge tube for second 100,000 g spin. After centrifugation, each pellet was resuspended in 150 µL HeBS plus Ca²⁺.

T Cell Proliferation Studies

To determine whether B cell-derived exosomes, with or without added OVA, could induce DO11.10 T cell proliferation, 100 µl of exosomes were added to 100,000 T cells per well in 100 µl of exosome-free CRPMI and plated in 96 well plates. After 96 hours of growth, cells were pulsed with 1 µ Ci/well of [H3]-thymidine (Perkin Elmer) for 24 hrs. Thymidine incorporation was then determined by harvesting the plates using a filtermate cell harvester onto GFC plates. Plates were then dried for approximately two hours. 25 µl of scintillation fluid was then added and counts were determined using the TopCount Plate Counter (Perkin Elmer, Waltham, Massachusetts).

Electron Microscopy

Exosome samples in PBS were diluted from an initial volume of 150 µL to a total volume of 1 mL. For the negative staining, exosomes were placed on formvar-coated 150-mesh copper grids. A drop of the undiluted exosome preparation was placed on a clean parafilm surface. The grid, formvar side to the sample, was then placed on top of the drop of sample and allowed to rest for 1 minute. The grid was then picked up and the excess liquid was wicked off with filter

paper touched to the edge of the grid. Next a drop of the stain was placed on the parafilm and the grid was put on top of it for 1 minute. After 1 minute, the grid was picked up, and the excess stain was wicked off. The grid was then allowed to dry completely before scoping. Various times were tested, from 30 seconds to 2 minutes and with rinsing after steps, as well as fixation with glutaraldehyde. Final developing solutions were tested: 1% Uranyl acetate, which gave a grainy image, and 1% Phosphotungstic acid at pH 7.0, which gave a much smoother stain around the exosomes.

The grids were viewed with the JEOL JEM-1230 Transmission Electron Microscope, JEOL USA, Inc., Peabody, MA. Images were taken with the Gatan UltraScan 4000 CCD camera, Gatan, Inc., Pleasanton, California.

All electron microscopy was performed at the VCU Department of Anatomy and Neurobiology Microscopy Facility supported, in part, with funding from NIH-NINDS Center core grant (5P30NS047463).

Airway Hyperresponsiveness or Induced Asthma

Airway hyperresponsiveness was induced using a mast cell/IgE dependent model, previously described by Dr. Stephen Galli.⁴⁷ The mice were sensitized by the intraperitoneal injection of 50 µg of OVA in 100 µL of saline on days 1, 3, 5 and 9. Then on day 22 the mice were challenged intranasally with (i) exosomes plus 200 µg of OVA in 20 µL of PBS, (ii) exosomes carrying OVA peptide (ExoPep), (iii) ExoPep plus 200 µg of OVA, or (iv) 200 µg of OVA in 20 µL of PBS and again on day 25 and 28. After the final OVA challenge (day 29), the mice were sacrificed. Bronchoalveolar lavage fluid (BALF) was collected by lavaging the lungs with 1 ml PBS. The BALF was centrifuged and supernatant fluids were saved for further

analysis. Pelleted cells were resuspended in 100 μ L PBS, counted, cytospun onto slides and stained with Diff-Quik (Siemens Healthcare Diagnostics, Deerfield, Illinois). Percentages were determined by counting of at least 200 cells per slide.

In addition, cardiac puncture was performed to collect serum for total IgE, OVA-specific IgE and OVA-specific IgG1 measurements. The heart was then perfused with 10 mL of PBS to flush the lungs of blood. The lungs and heart were removed and fixed in 10% formalin (Fisher) and stored at 4°C until paraffin sections could be prepared, if appropriate, by the Anatomic pathology research service at VCU.

Statistical analysis

All statistics were done using the student's two-tailed T-test.

V. Results

In 2011, Heyman, *et al.*, reported an extensive study of the interrelationship among B cells, dendritic cells (CD11c⁺) and T cells.⁴⁸ In the study, CD11c⁺ cells primed *in vivo* with OVA-IgE complexes were able to induce specific T cell proliferation, whereas CD19⁺ B cells could not. While the authors concluded that the data suggested a scenario where CD19⁺/CD23⁺ B cells transport IgE-antigen to the spleen where the antigen is captured and presented to CD4 T cells by CD11c⁺ cells, they were not able to directly demonstrate the delivery of antigen from B cells to CD11c⁺ cells. The authors noted that antigen could be delivered to T cells by several means including pinocytosis, trogocytosis, endocytosis or exosomal transfer.

Our laboratory previously published a paper indicating that ADAM10 is required for CD23 incorporation into exosomes.⁴⁹ My project was to repeat several of these experiments, to further characterize exosomes derived from murine B cells, and to determine whether IgE/OVA complexes carried by exosomes could provide an effective means of delivering antigen to stimulate T cell proliferation.

A. B Cell Exosomes Carry Surface CD23

B cells were cultured in exosome free CRPMI under four conditions: (1) 1 µg/mL anti-CD40 alone; (2) 1 µg/mL anti-CD40 and 10,000 units IL-4; (3) 1 µg/mL anti-CD40 and 50 µg/mL IgE/DNP; and (4) 1 µg/mL anti-CD40, 10,000 units IL-4, and 50 µg/mL IgE(conjugated to either DNP or TNP). As shown on Figure 14, B cells stimulated with IL-4- express CD23 at higher levels than cells not having such stimulation.

B. CD23 Positive B Cells May Possess the Ability to Transport IgE

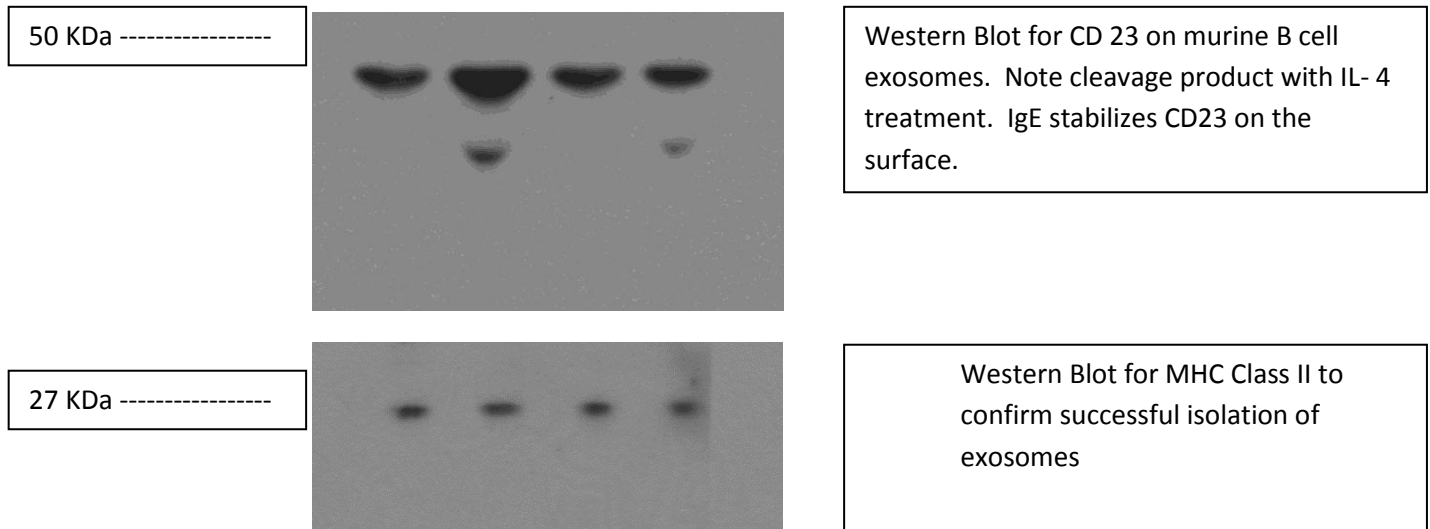
To assess observe the ability of CD23⁺ exosomes to act as APCs to T cells, several studies were conducted to determine whether the exosomes could bind IgE and IgE/OVA complexes. The isolated exosomes were passed through SDS Page 10% Bis-Tris gels and then transferred to nitrocellulose paper. The sample was incubated with and without IgE-DNP in the lanes indicated with Acris anti-IgE antibody as the primary antibody and goat anti-rabbit HRP as the secondary antibody. As shown on Figure 15 and Figure 16, there are several bands indicating not only the presence of IgE, but several fragments with smaller bands as IgE displays

fragments of lesser size.⁵⁰ This would appear to indicate that it is possible for CD23 molecules on the surface of exosomes to bind and transport IgE molecules.

C. Anti-CD40-Stimulated B Cells Release Greater Amounts of CD23 Positive Exosomes Than LPS-Stimulated Cells

LPS is one of the best studied immunostimulatory components of bacteria and, with excessive signaling, can induce systemic inflammation and sepsis.⁵¹ LPS is an important structural component of the outer membrane of Gram-negative bacteria. Stimulation of B cells by LPS through the toll-like receptor (TLR) 4 pathway induces B-cell proliferation, antibody secretion, and promotes B cells to function as antigen-presenting cells by enhancing expression of MHC class II and co-stimulatory molecules.⁵² B cells, therefore, are an important bridge between the innate and the adaptive immune system, due to their ability to be activated by pathogen-associated molecules like LPS and to generate antigen-specific antibodies. Another study, Figure 17, indicated that murine B cells co-incubated with CD-40L stimulated CD23⁺ exosome release at an amount greater than LPS-stimulated cells. This result probably is caused by anti-CD40 directly stimulates all B cells, whereas LPS utilizes the TLR4 pathway and stimulates less than 50% of all B cells.

Figure 14

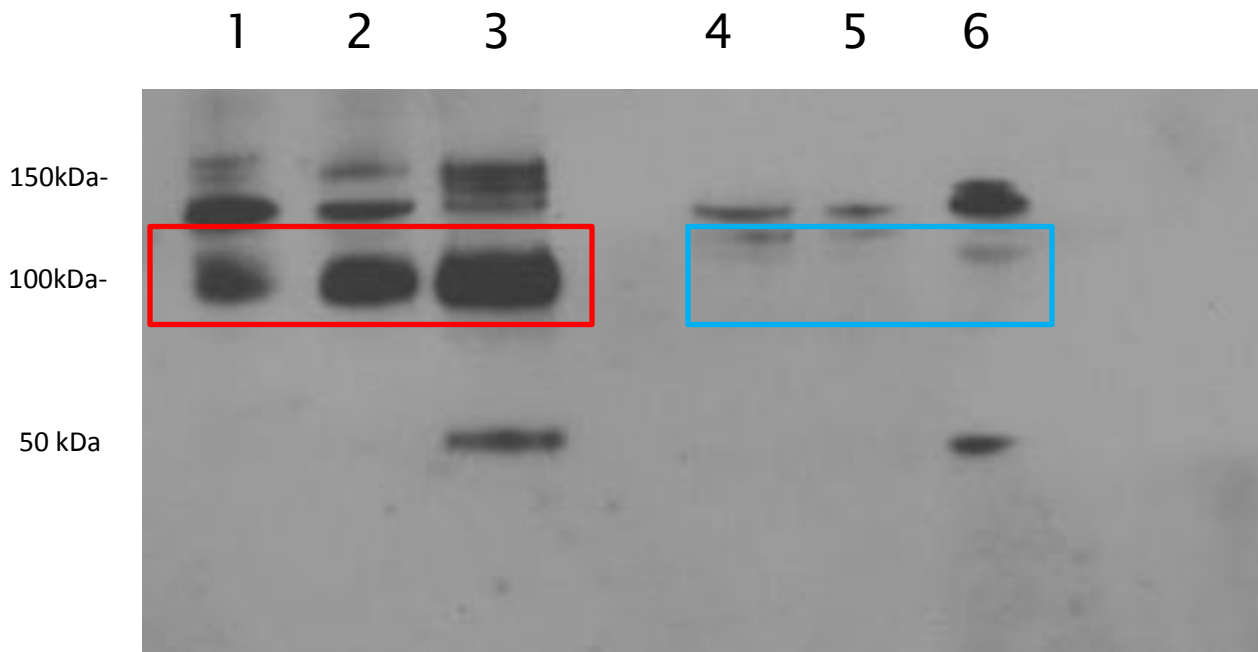


B cells stimulated with
 Lane 1-anti-CD40
 Lane 2-anti-CD40+IL-4
 Lane 3-anti-CD40+IgE
 Lane 4-anti-CD40+IL-4+IgE
 Detection of CD23 on Murine B cell exosomes

B cells were cultured in exosome free CRPMI under four conditions: (1) 1 μ g/mL anti-CD40 alone; (2) 1 μ g/mL anti-CD40 and 10,000 units IL-4; (3) 1 μ g/mL anti-CD40 and 50 μ g/mL IgE/DNP; and (4) 1 μ g/mL anti-CD40, 10,000 units IL-4, and 50 μ g/mL IgE(conjugated to either DNP or TNP). Exosomes were then isolated after several centrifugation steps.

Western Blots were performed 10% Bis-Tris gels for samples. 20 μ g of sample per lane was pipetted into the gel and the apparatus ran for 35 minutes at 200V, 120mA. Transfer of the samples on to nitrocellulose paper was accomplished using a BioRad for 1.5 hours at 100V, 200mA or overnight in a cold room at 25V. For CD23 Western blots, CD23 was detected with rabbit anti-mouse CD23 (anti- LZ) followed by goat anti-rabbit HRP antibodies. For MHC class II blots, the proteins were detected using rabbit anti-H2-I/Ad β followed by goat anti-rabbit HRP-conjugated secondary antibodies.

Figure 15



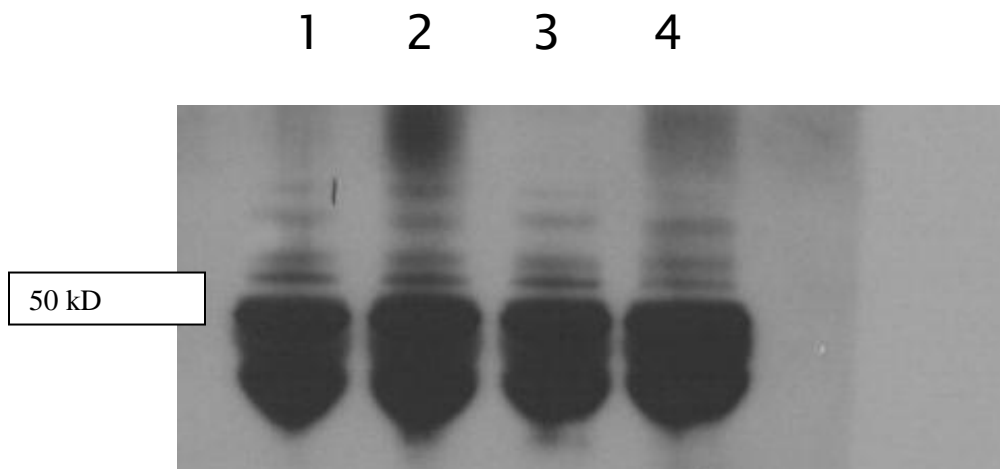
Lane 1 WT Balb/c B cells + IL4 + anti-CD40
Lane 2 WT Balb/c B cells + IL4 + anti-CD40 + IgE-DNP
Lane 3 WT Balb/c B cells + IL4 + anti-CD40 + IgE-DNP + OVA-DNP
Lane 4 CD23 K/O+ IL4 + anti-CD40
Lane 5 CD23 K/O+ IL4 + anti-CD40+ IgE-DNP
Lane 6 CD23 K/O+ IL4 + anti-CD40+ IgE-DNP + OVA-DNP

Western Blot of exosomes with IgE bound

The isolated exosomes were passed through SDS Page 10% Bis-Tris gels and then transferred to nitrocellulose paper. The sample was incubated with IgE-DNP in the lanes indicated, with Acris AP21485AF-N, (diluted 1:500/1000) as the primary antibody against IgE and Goat anti-rabbit HRP as the secondary antibody.

Red box indicates the heavy chain of IgE, whereas the blue box notes the absence of IgE. There is some cross-reactivity between the Acris antibody and Ova (44kDa band, in lanes 3 and 6).

Figure 16



Lane 1 WT Balb/c B cells + IL4 + anti-CD40

Lane 2 WT Balb/c B cells + IL4 + anti-CD40 + IgE-DNP + OVA-DNP

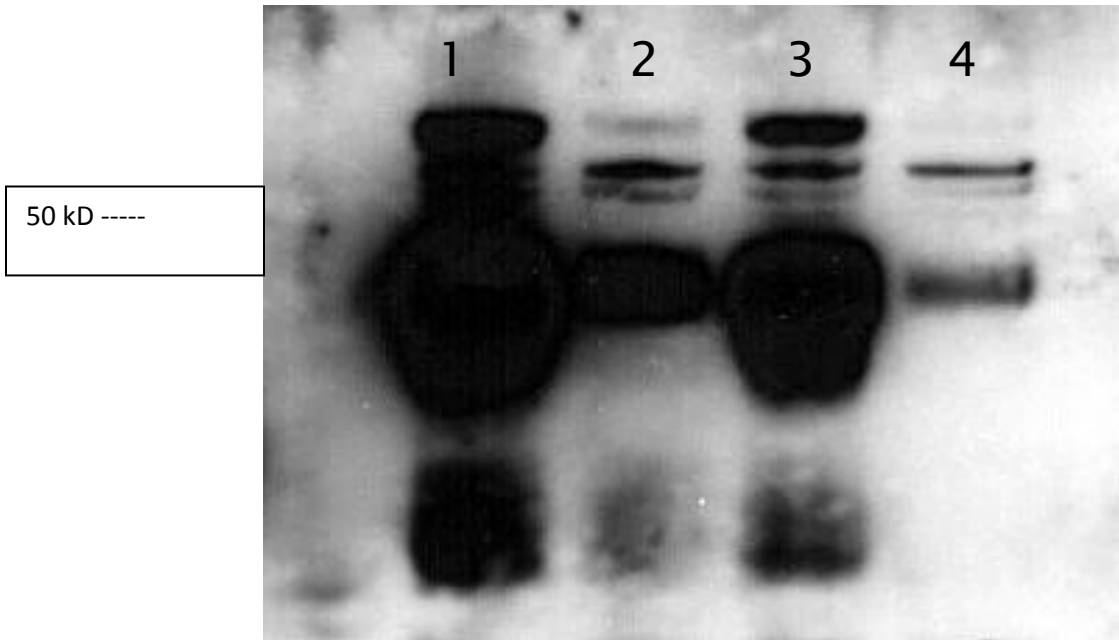
Lane 3 WT Balb/c B cells + IL4 + anti-CD40 + IgE-DNP

Lane 4 WT Balb/c B cells + IL4 + anti-CD40 + OVA-DNP

IgE and CD23 on B cell exosomes

Simultaneous antibody incubation for CD23 and IgE.

Figure 17



Lane 1 ADAM10^{-/-} stimulated with anti-CD40, IgE-TNP and IL-4

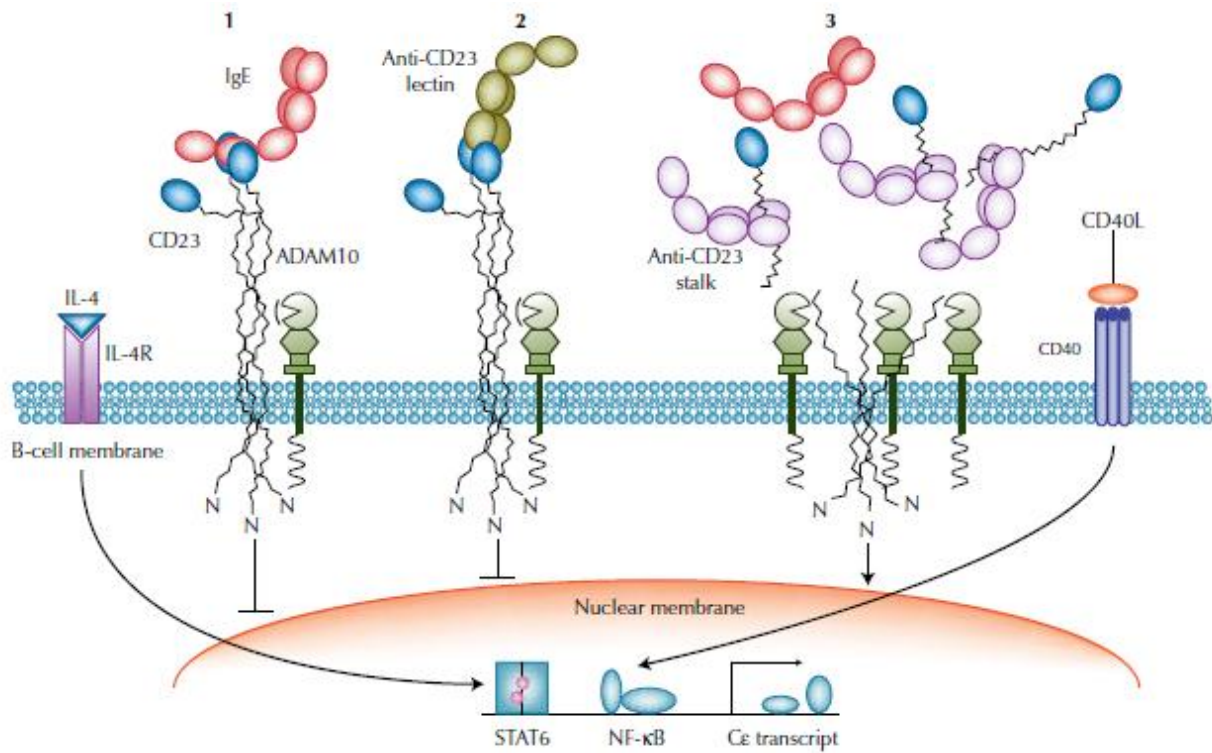
Lane 2 ADAM10^{-/-} stimulated with LPS, IgE-TNP and IL-4

Lane 3 WT stimulated with anti-CD40, IgE-TNP and IL-4

Lane 4 WT stimulated with LPS, IgE-TNP and IL-4

5, 000,000 B cells isolated from WT and ADAM10^{-/-} mice stimulated with anti-CD40 (1µg/mL) or LPS (1µg/mL), plus IgE-TNP (50 µg/5mL) and IL-4 (10,000 units). CD23 was detected with rabbit anti-mouse CD23 (anti- LZ) followed by goat anti-rabbit HRP antibodies.

Figure 18



IgE Synthesis Pathway

IL-4 and CD40 ligation are two of the primary signals for IgE synthesis. In addition, anti-CD40, present on TH cells provides the necessary signal for B cell activation.

Conrad, D.H., Ford, J.W., Sturgill, J.L., Gibb, D.R. CD23: an overlooked regulator of allergic disease. *Curr. Allergy Asthma Rep.* 7(5):331-7 (2007).

D. B Cell Exosomes Present CD80 and CD86 on their Surface

As noted previously, activated B cells up regulate various costimulatory molecules such as CD80 (B7.1) and CD86 (B7.2) that are ligands for CD28 on T cells which induce the T cell to produce cytokines that induce B cells to switch isotypes and differentiate to produce antibodies, such as IgE.⁵³ A Western Blot was performed to determine whether the exosomes being studied also present CD80 and CD86, and, if so, whether their expression on the surface of exosomes differed. As shown on Figure 19, CD86 is expressed in greater amounts than CD80.

E. Exosomes Can Be Separated Into CD23 Positive and CD23 Negative Populations

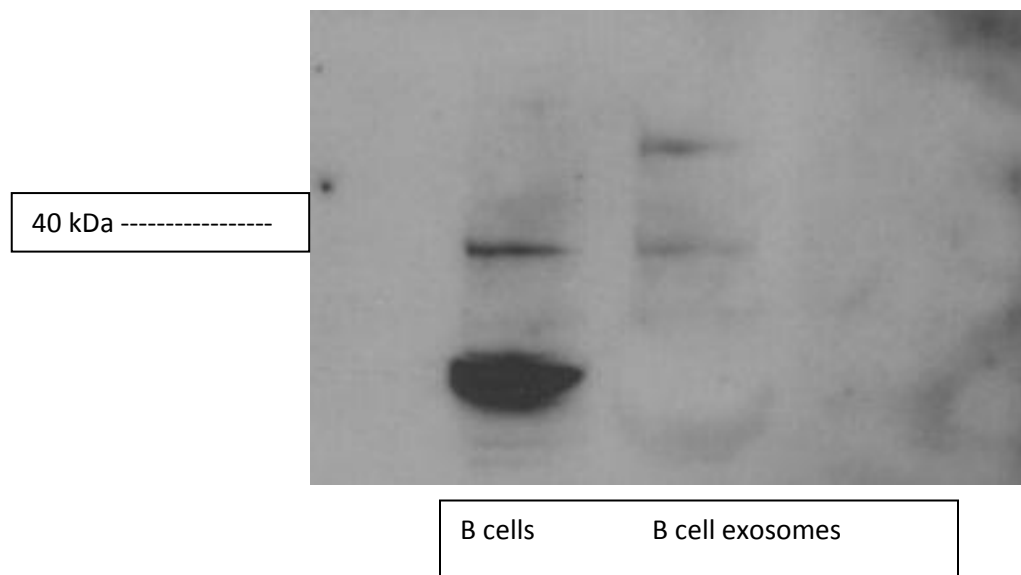
Using IgE coupled to Affi-Gel, as previously described, several experiments were conducted to determine whether B cell-derived exosomes could be separated into CD23 positive and CD23 negative subsets. Figure 20 is a depiction of the steps taken during the process of isolating B cell-derived exosomes and subsequently separating the exosomes into CD23 positive and CD23 negative subsets. As shown in Figure 21, such a separation is feasible. The ability to separate exosomes into differing subsets based upon surface receptor expression underscores the fact that exosomes are not a homogeneous population.

F. CD 23 is Expressed in ADAM10 K/O Mice

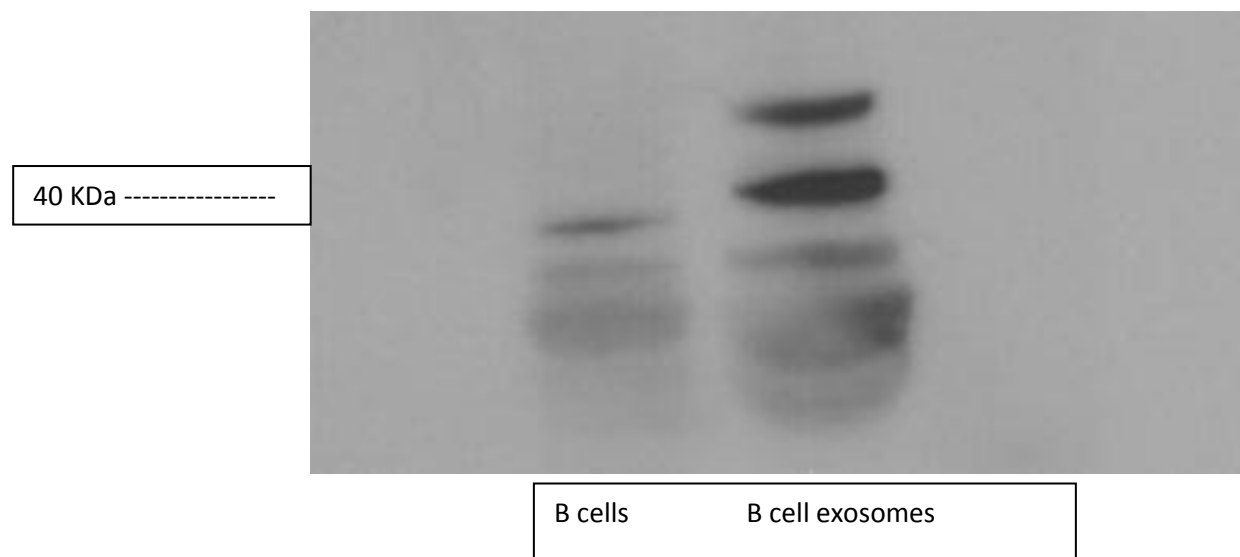
Our laboratory previously has shown that ADAM10 binding of CD23 was essential for sorting into exosomes. As shown on Figure 22, exosomes from B cells stimulated with IL-4 and anti-CD40 do express CD23, albeit at lower levels than wild type mice. It is possible that another ADAM, such as ADAM17, compensate for the loss of ADAM10 thereby providing for CD23 exosomal sorting.

Figure 19

CD80 Expression



CD86 Expression



Expression of CD80 and CD86 on B cell exosomes

Figure 20

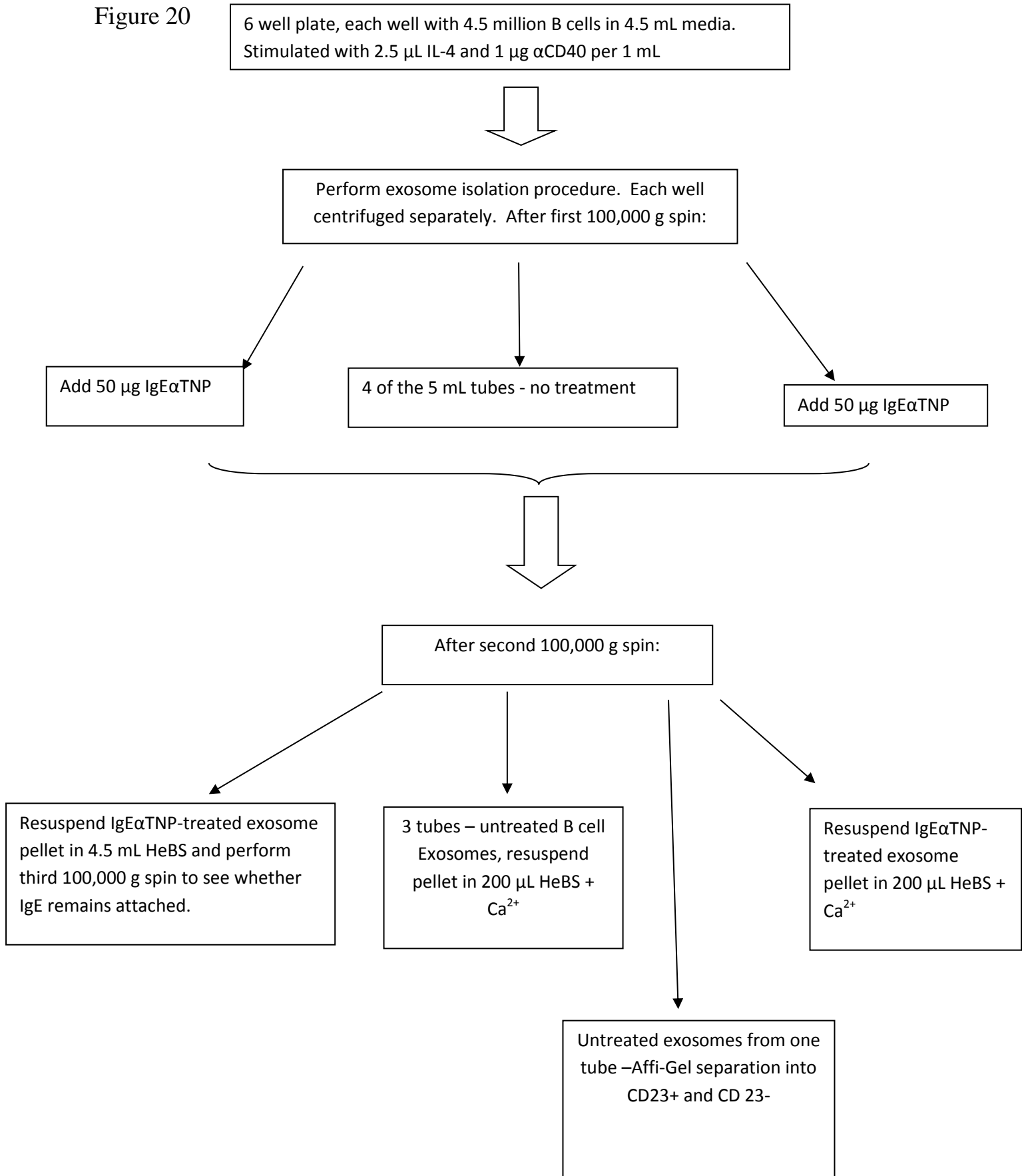
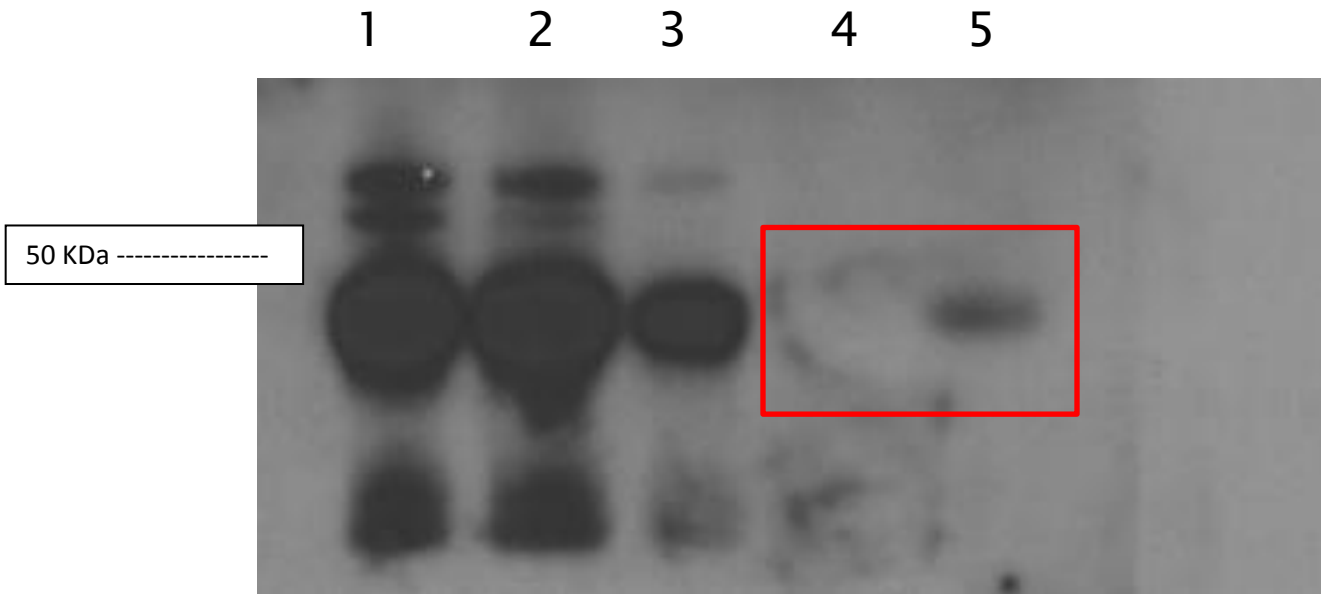


Figure 21



Lane 1 WT B cell exosomes

Lane 2 WT B cell exosomes + IgE-TNP, after second 100,000 x g spin

Lane 3 WT B cell exosomes+ IgE-TNP, after third 100,000 x g spin

Lane 4 WT B cell exosomes, from Affi-Gel retained supernatant – CD23 negative, no band

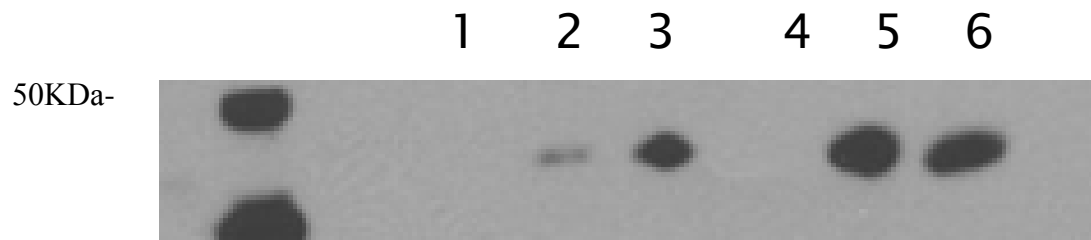
Lane 5 WT B cell exosomes, from Affi-Gel retained pellet – CD23 positive band

Western Blot of IgE-coupled Affi-Gel separation of murine B cell exosomes

In brief, in a 6 well plate, each well with 4.5 million B cells in 4.5 mL media. Each well was stimulated with 20,000 units of IL-4 and 1 μ g α CD40 per 1 mL. Exosomes were isolated using the previously described centrifugation procedure. However, after first 100,000 g spin, some samples were given 50 μ g IgE-TNP, some samples received no additional treatments, but were subjected to the Affi-Gel separation procedure and to determine whether exosomes could be divided into CD23+ and CD 23- populations. Other exosomes were subjected to a third 100,000 x g spin to see whether IgE-TNP remained attached (data not shown).

CD23 was detected with rabbit anti-mouse CD23 (anti- LZ) followed by goat anti-rabbit HRP antibodies.

Figure 22



B cells incubated with:

1-WT, anti-CD40

2-WT, anti-CD40 and IL-4

3-WT, anti-CD40, IL-4 and IgE

4-AD10KO, anti-CD40

5-AD10KO, anti-CD40 and IL-4

6-AD10KO, anti-CD4-, IL-4 and IgE

CD 23 is Expressed On ADAM10 K/O Mice

Although ADAM10 is important in the sorting of CD23 into exosomes, exosomes from B cells stimulated with IL-4 and anti-CD40 do express CD23, albeit at lower levels than wild type mice. It is possible that another ADAM, such as ADAM17, compensate for the loss of ADAM10 thereby providing for CD23 exosomal sorting.

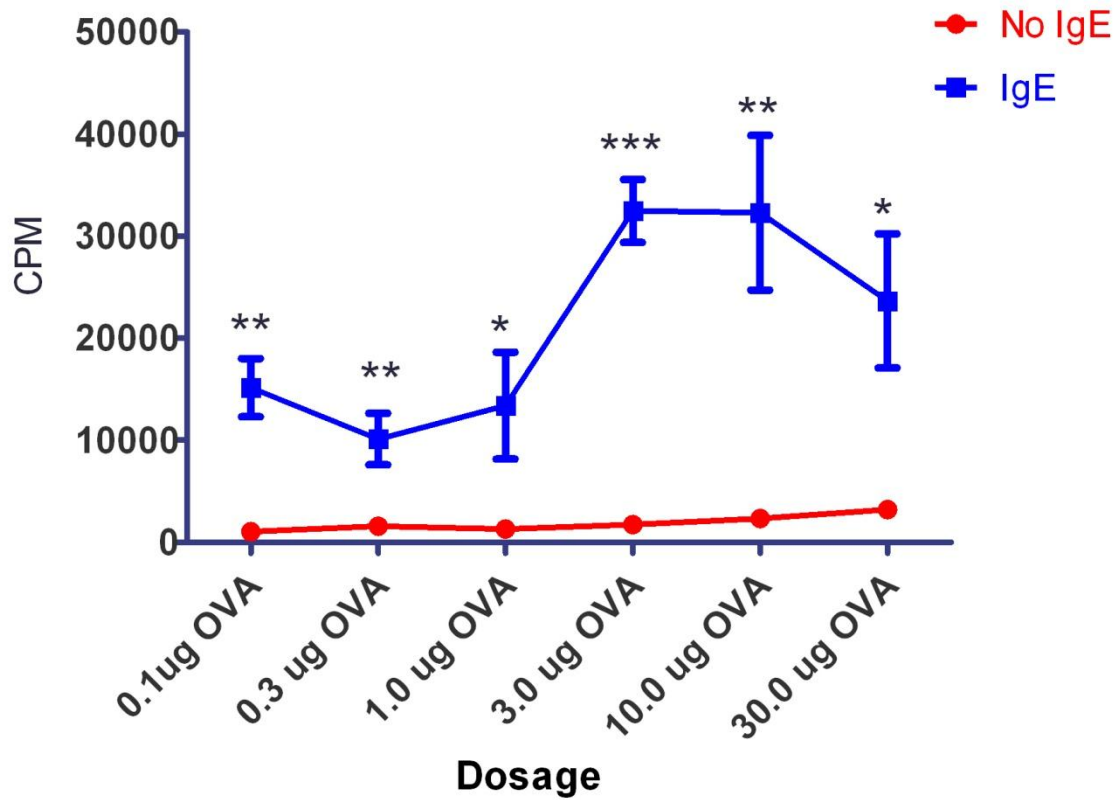
G. Exosomes Can stimulate T cell proliferation

DO11.10 (Balb/c) and OT-II T (C57BL/6) transgenic mice express a TCR that is specific for OVA 323–339 even though the MHC backgrounds of the mice are different.⁵⁴ Figure 23 is a graph of T cell proliferation at various doses of OVA added to Balb/c mouse B cell culture, wherein the IgE⁺/IgE⁻ B cells take up the protein and the B cell exosomes carry the OVA peptide for presentation to DO11.10 T cells (specific for an OVA peptide). Figure 24 is a graph of T cell proliferation at various doses of OVA added to C57/B6 B cell culture for presentation to OTII T cells (specific for OVA, but on a C57/B6 background). In each case, prior to isolating T cells, the splenocytes were treated with B220 beads to remove B cells, CD11b beads to remove monocytes, and CD11c beads to remove dendritic cells before treated with L3T4 beads to select for T cells.

As evident from the graphs, Balb/c mouse T cells proliferate at a much greater amount than those of C57/B6 mice. This is consistent with other studies indicating that BALB/c mice represent a relatively hyperresponsive strain (T_H2 prone) as compared to C57BL/6 mice, which are generally considered as low T_H2 responders (T_H1 prone) as they display low airway responsiveness in response to an allergen.⁵⁵

Figure 23

Balb/c mice -- DO11.10



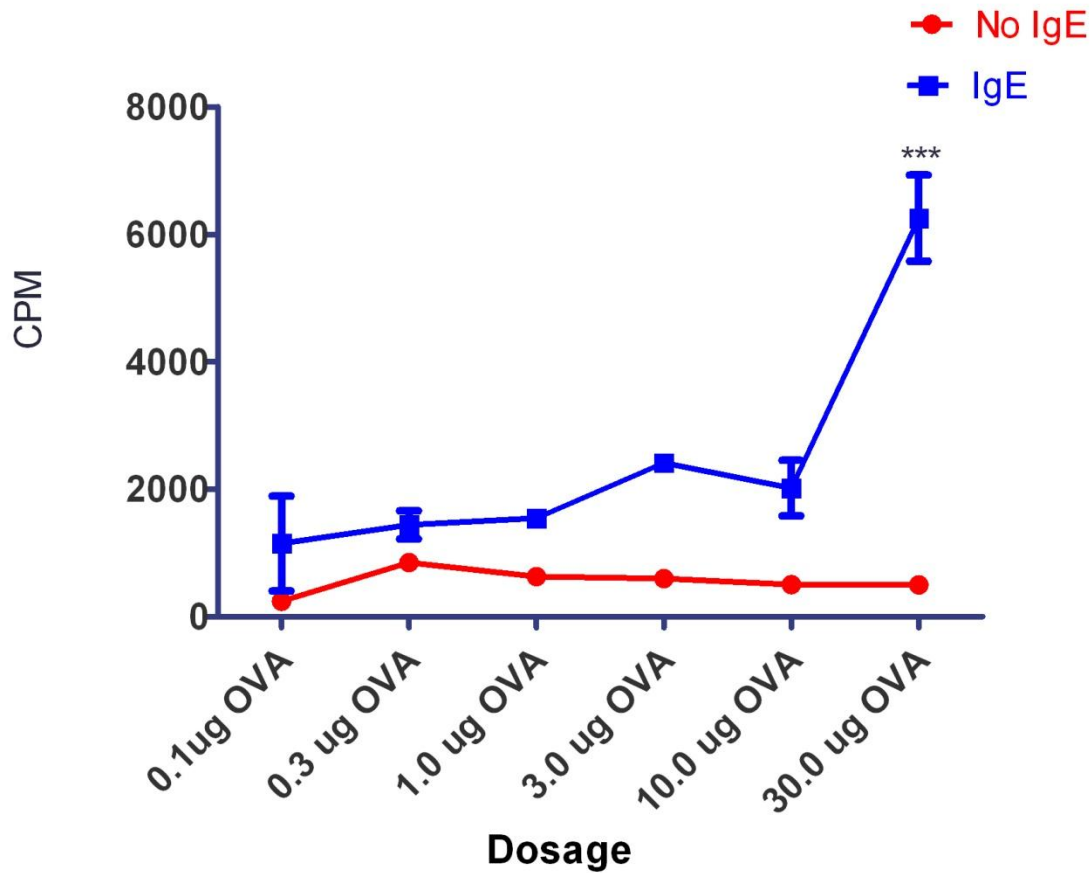
Student's T-test */**/*** $P < .05$, $.005$, or $.0001$, respectively.

T cell proliferation in Balb/c mice

A graph of T cell proliferation at various doses of OVA added to Balb/c mouse B cell culture, wherein the IgE+/IgE- B cells take up the protein and the B cell exosomes carry the OVA peptide for presentation to DO11.10 T cells.

Figure 24

C57/B6 mice - OTII



Student's T-test *** $P < .0001$

T cell Proliferation in C57/B6 mice

A graph of T cell proliferation at various does of OVA added to C57/B6 B cell culture for presentation to OTII T cells.

Figure 25 illustrates the influence of dendritic cells on the presentation of OVA peptide by exosomes. On Day 1, B cells were isolated and plated the cells at 1 million per 1 mL media (total 5 million B cells in 5 mL media) in a 6 well plate with IgE/OVA complexes (50µg/20µg per 5 mL) for a 3 day culture before isolating exosomes. On Day 2, CD11c⁺ dendritic cells were isolated and plated in 6 well plates. To one-half of the DCs, 1µg/mL LPS was added for stimulation and maturation of the DCs over 24 hours; the other half were untreated. Finally, T cells (100,000), dendritic cells (50,000) and exosomes (100 µL) per 200 µL well were plated in various combinations to separate wells of 96 well plates. The plates were incubated for 3 days before T cell proliferation was measured by the incorporation of tritiated thymidine. Surprisingly, the exosomes from B cells incubated with OVA induced greater T cell proliferation than the proliferation induced by the addition of stimulated DCs.

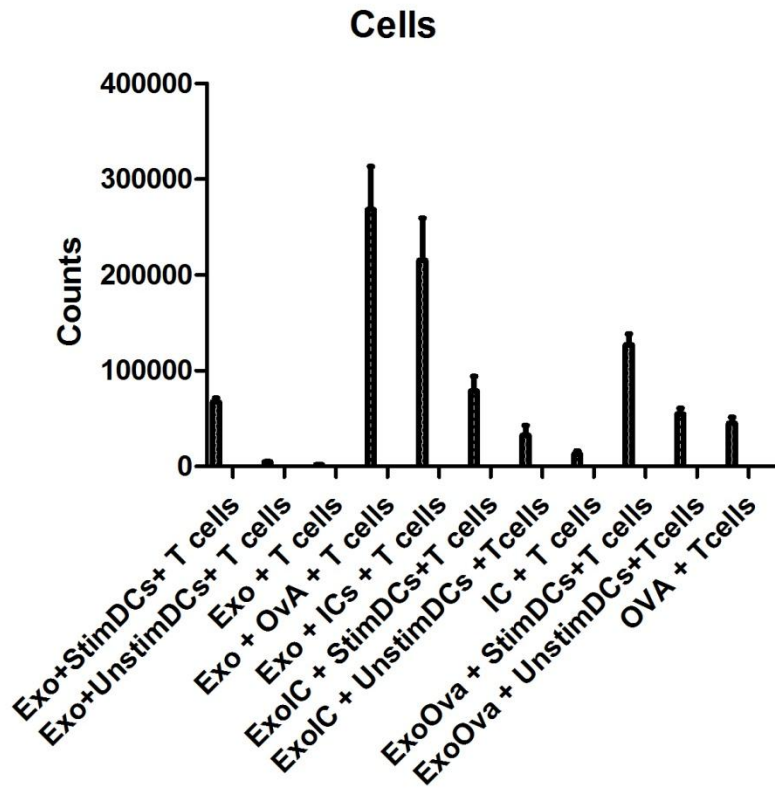
Figure 26 illustrates the critical importance of IgE on the ability of B cell-derived exosomes to carry antigen and thereby induce T cell proliferation. On Day 1, B cells were isolated and plated the cells at 1 million per 1 mL media (total 5 million cells in 5 mL media) in a 6 well plate. One subset, the IgE positive subset, was treated with IgE-TNP (50 µ g total), 1 µg/mL anti-CD40 and 10,000 units IL4 for 24 hours. Another subset, the IgE negative subset, received only 1 µg/mL antiCD40 and 10,000 units IL4 for 24 hours. The next day the cells were washed and 5 µg IgE-TNP was added to the IgE positive subset. Fresh IL4 and antiCD40 were added to both the IgE positive and IgE negative subsets. After four hours, OVA-TNP was added in in the amounts noted for 24 hours before isolating exosomes. T cells (100,000 in 100 µL) and exosomes (100 µL out of the total 1mL filtered volume of exosomes produced by 5 million B cells) per 200 µL well were plated in groups of five per dosage of OVA to 96 well plates. The

plates were incubated for 3 days before T cell proliferation was measured by the incorporation of tritiated thymidine. As evident from Figure 27, exosomes incubated with IgE prior to the introduction of OVA-TNP effectively induce T cell proliferation, whereas B cell exosomes that have not been exposed to IgE do not.

As noted previously, there is a distinct population of CD23 positive exosomes that can be isolated from B cells. To further this finding, exosomes were isolated from CD23^{-/-} (CD23 knockout) mice to determine whether the addition of IgE could compensate for the absence of CD23. As in other experiments 1 million B cells per 1mL (5 million cells total) were cultured with anti-CD40 (1µg/mL), IL-4 (10,000 units per 5mL). One WT and one CD23^{-/-} group received 10µg/mL of IgE and after washing the cells 24 hours later those cells received a boost of 1µg/mL of IgE. After a 48 hour incubation, exosomes were isolated. OVA-TNP was added in in the amounts noted (.1 µg to 30 µg) for 24 hours before isolating exosomes. DO11.10 T cells (100,000 in 100 µL) and exosomes (100 µL out of the total 1mL filtered volume of exosomes produced by 5 million B cells) per 200 µL well were plated in groups of five per dosage of OVA to 96 well plates. The plates were incubated for 3 days before T cell proliferation was measured by the incorporation of tritiated thymidine.

As shown on Figure 28, only WT B cell exosomes (CD23 positive) that had received IgE induced a marked proliferation of DO11.10 T cells. Neither WT exosomes without IgE nor CD23 KO exosomes treated with IgE could induce T cell proliferation. This result further confirms the importance CD23 and IgE working in tandem to produce an immune response.

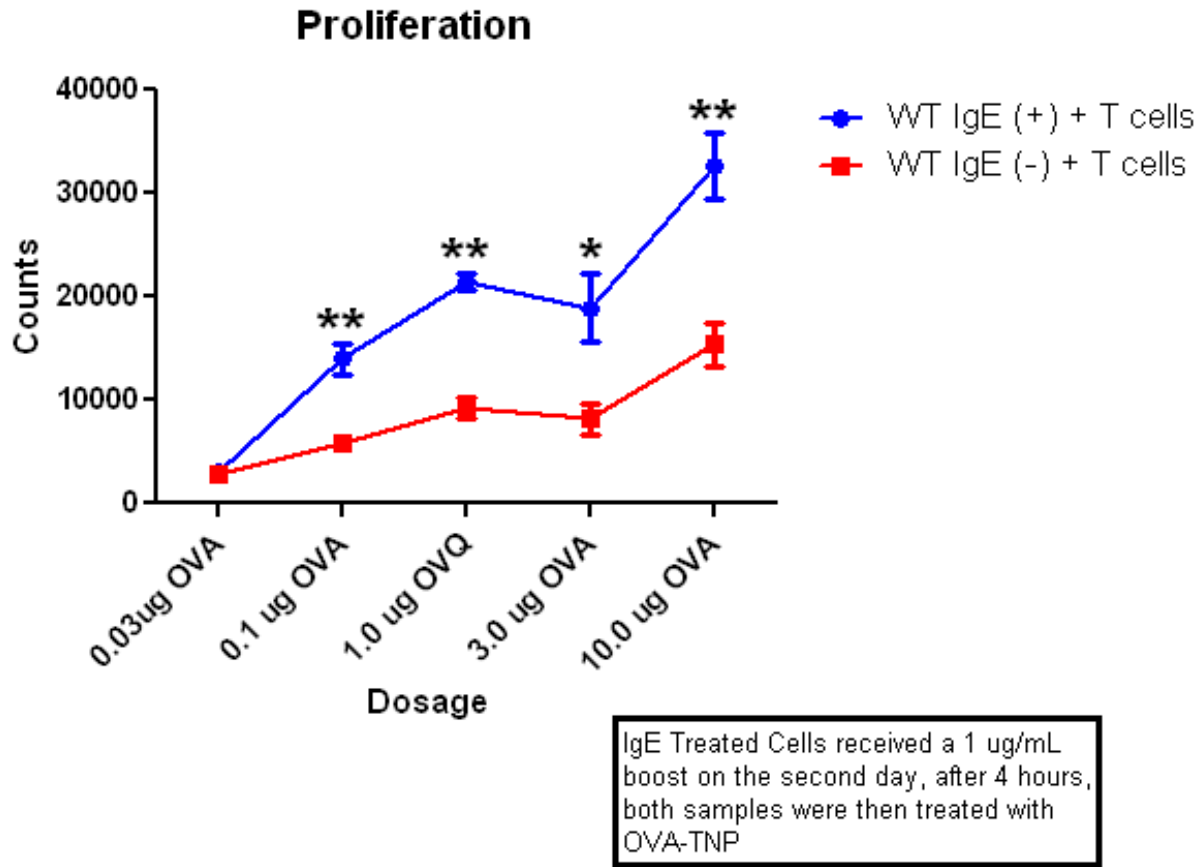
Figure 25



T cell Proliferation induced by Exosomes and Dendritic Cells

On Day 1, B cells were isolated and plated the cells at 1 million per 1 mL media in a 6 well plate with IgE/OVA complexes (50µg/20µg per 5 mL) for a 3 day culture before isolating exosomes. On Day 2, CD11c⁺ dendritic cells were isolated and plated in 6 well plates. To one-half of the DCs, 1µg/mL LPS was added for stimulation and maturation of the DCs over 24 hours; the other half were untreated. Finally, T cells (100,000), dendritic cells (50,000) and exosomes (100 µL) per 200 µL well were plated in various combinations to separate wells of 96 well plates. The plates were incubated for 3 days before T cell proliferation was measured by the incorporation of tritiated thymidine.

Figure 26

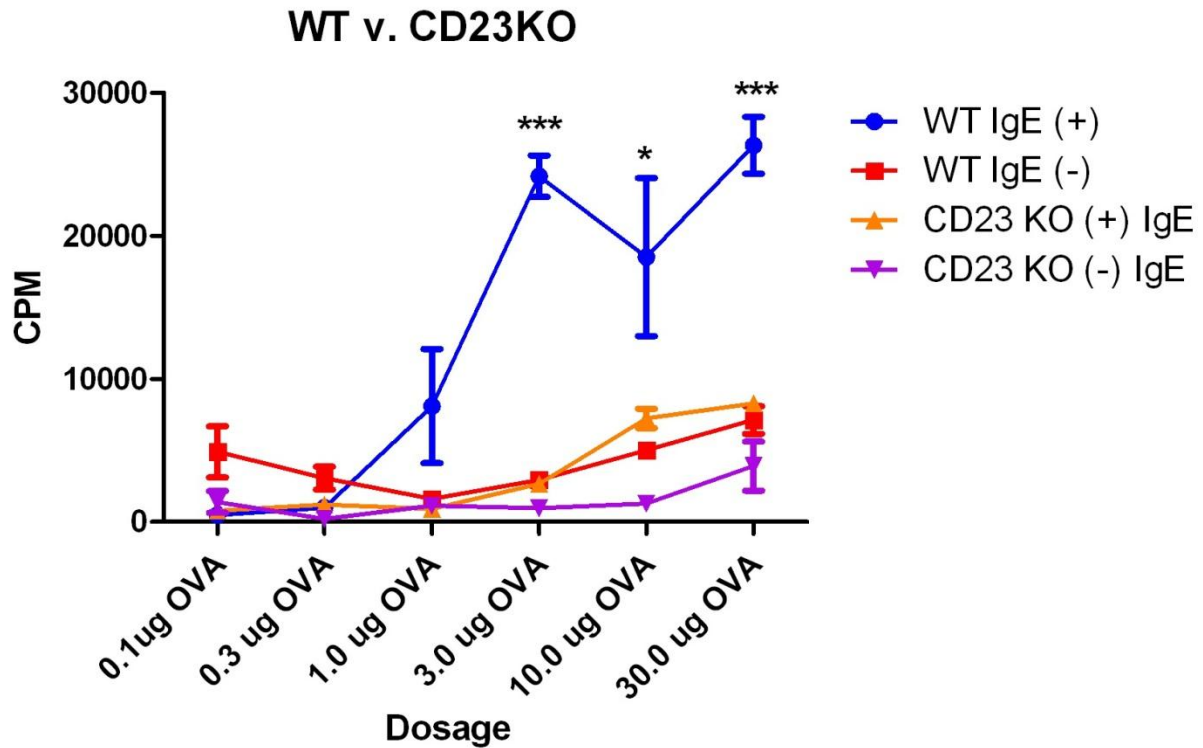


Student's T-test */** $P < .05$ or $.005$ respectively.

The Effect of IgE on Exosome-Induced T cell Proliferation

On Day 1, B cells were isolated and plated the cells at 1 million per 1 mL media (total 5 million cells in 5 mL media) in a 6 well plate. One subset, the IgE positive subset, was treated with IgE-TNP (50 μ g total), 1 μ g/mL anti-CD40 and 10,000 units IL4 for 24 hours. Another subset, the IgE negative subset, received only 1 μ g/mL antiCD40 and 10,000 units IL4 for 24 hours. The next day the cells were washed and 5 μ g IgE-TNP was added to the IgE positive subset. Fresh IL4 and antiCD40 were added to both the IgE positive and IgE negative subsets. After four hours, OVA-TNP was added in in the amounts noted for 24 hours before isolating exosomes. T cells (100,000 in 100 μ L) and exosomes (100 μ L out of the total 1mL filtered volume of exosomes produced by 5 million B cells) per 200 μ L well were plated in groups of five per dosage of OVA to 96 well plates. Exosomes incubated with IgE prior to the introduction of OVA-TNP effectively induce T cell proliferation, whereas B cell exosomes that have not been exposed to IgE do not.

Figure 27



Student's T-test */**/** $P < .05$, $.005$, or $.0001$, respectively.

CD23 and IgE Critical for T Cell Proliferation

WT B cell CD23 positive exosomes treated with IgE induced a marked proliferation of DO11.10 T cells. Neither WT exosomes without IgE nor CD23 KO exosomes treated with IgE could induce T cell proliferation.

After showing that B cell-derived exosomes could be separated into separate subpopulations of CD23⁺ and CD23⁻ exosomes, an experiment was undertaken to see whether the CD23⁺ would induce a greater amount of DO11.10 T cell proliferation than CD23⁻ exosomes. In this study B cells were isolated from mouse spleens and incubated with anti-CD40 (1 µg/mL) and IL-4 (10,000 units) for 3 days. The exosomes were isolated and separated into CD23⁺ and CD23⁻ populations as previously described. Fifteen minutes prior to the second 100,000 x g spin, each of the CD23⁺ and CD23⁻ samples further were treated as follows: (1) exosomes only; (2) immune complexes (50 µg IgE-TNP and 20 µg OVA-TNP); (3) 50 µg IgE-TNP; and (4) 20 µg OVA-TNP. The exosomes were diluted to 1mL of cRPMI and sterile filtered. 100 µL of exosomes were added to 100,000 previously stimulated DO11.10 T cells (in 100 µL of cRPMI). The T cells had been stimulated with anti CD3 (2C11) and CD28 overnight per the BioLegend Mouse T Cell Activation with anti-CD3ε (clone 145-2C11) Protocol (<http://www.biolegend.com/support/prot>)

As shown on Figure 28, CD23⁺ treated with immune complexes or OVA-TNP induced greater T cell proliferation than CD23⁻ exosomes. In fact, only CD23⁻ exosomes treated with OVA-TNP induced proliferation, which may be due solely to the OVA-TNP and not to an exosomal effect.

H. Experiment to Determine Whether Exosomes Derived from B Cells Cultured With OVA Could Induce Airway Hypersensitivity

Figure 29 is a depiction of the mast cell dependent model of AHR, with modifications noted to illustrate four treatment conditions. In brief, Balb/c mice were sensitized by the i.p. injection of 50 µg of OVA in 100 µL of saline on days 1, 3, 5 and 9. On day 22 the

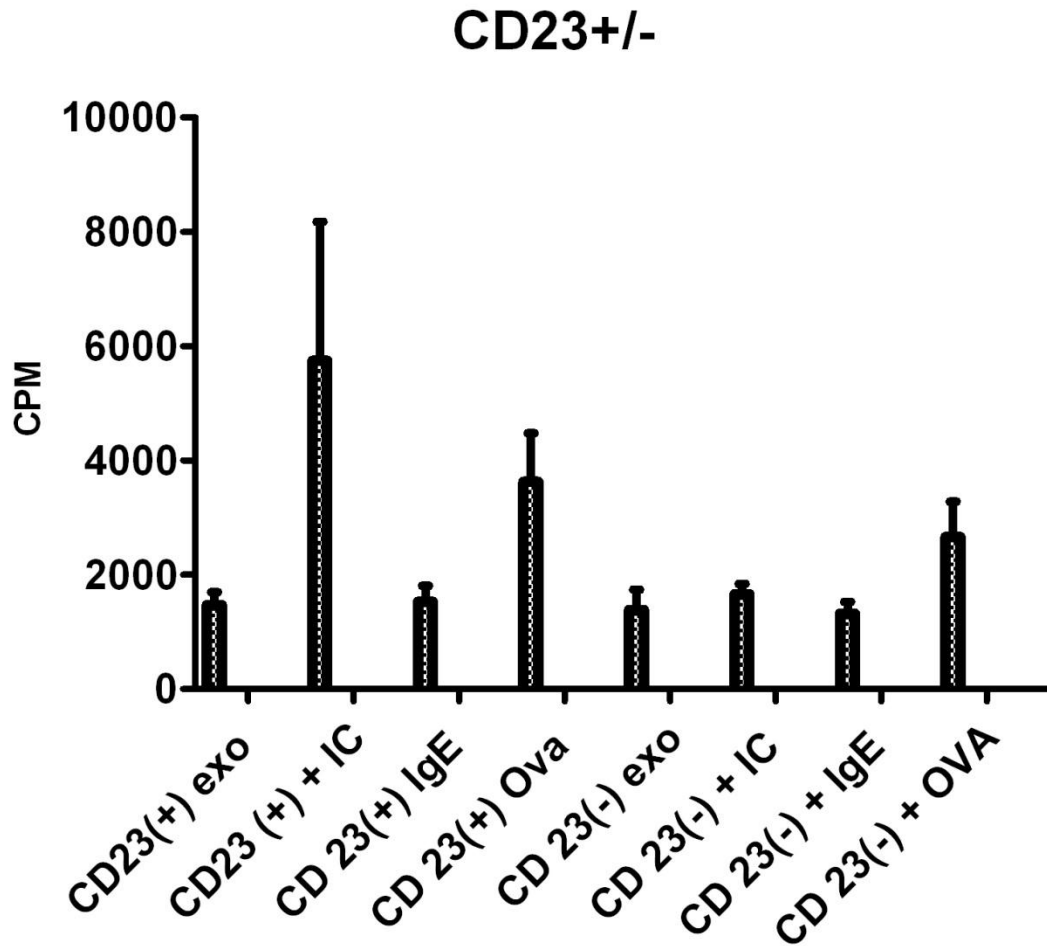
mice were challenged i.n. with (i) exosomes plus 200 µg of OVA in 20 µL of PBS, (ii) exosomes carrying OVA peptide (ExoPep), (iii) ExoPep plus 200 µg of OVA, or (iv) 200 µg of OVA in 20 µL of PBS and again on day 25 and 28. After the final OVA challenge (day 29), the mice were sacrificed. BALF was collected by lavaging the lungs and then centrifuged. Pelleted cells were resuspended in 100 µL PBS, counted, cytopun onto slides. Cells were counted by visual inspection. Percentages were determined by counting of at least 200 cells per slide.

As evident from the cell counts set forth in Figure 30, B cell derived exosomes cultured with OVA (ExoPep) alone were not sufficient to induce AHR in Balb/c mice. OVA-sensitized mice or mice receiving OVA in conjunction with exosomes did exhibit AHR, but this result probably was due to the addition of OVA and not to the exosomes.

I. Transmission Electron Microscope Picture of B Cell Exosomes

After isolating B220 B cell derived exosomes, several samples that had not been incubated with immune complexes or with OVA were reserved and used for electron microscopy. Figure 31 is a representative example of the exosomes isolated for the studies discussed herein.

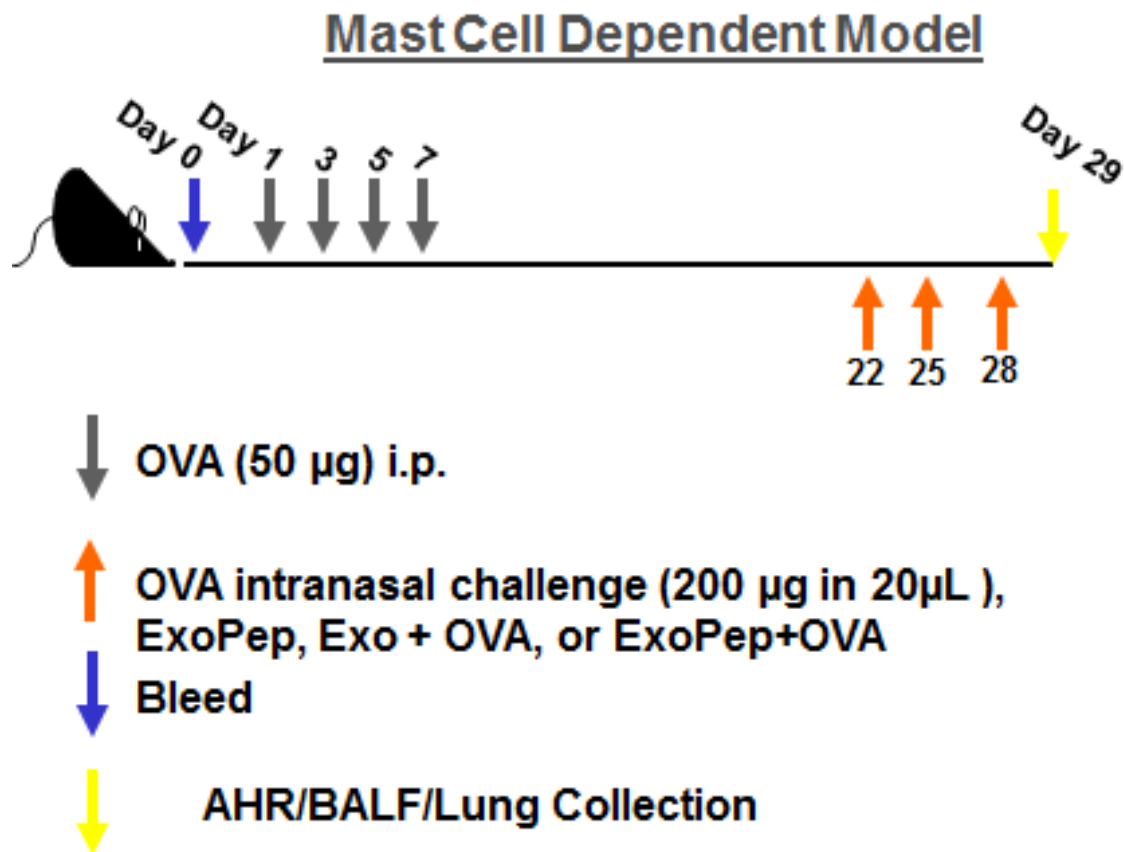
Figure 28



CD23⁺ Exosomes Induce Greater T Cell Proliferation Than CD23⁻ Exosomes

CD23⁺ and CD23⁻ exosome populations were separated and then treated prior to the second 100,000 x g spin with: (1) exosomes only; (2) immune complexes (50 μ g IgE-TNP and 20 μ g OVA-TNP); (3) 50 μ g IgE-TNP; and (4) 20 μ g OVA-TNP. The exosomes were plated with DO11.10 T cells and then assessed for proliferation.

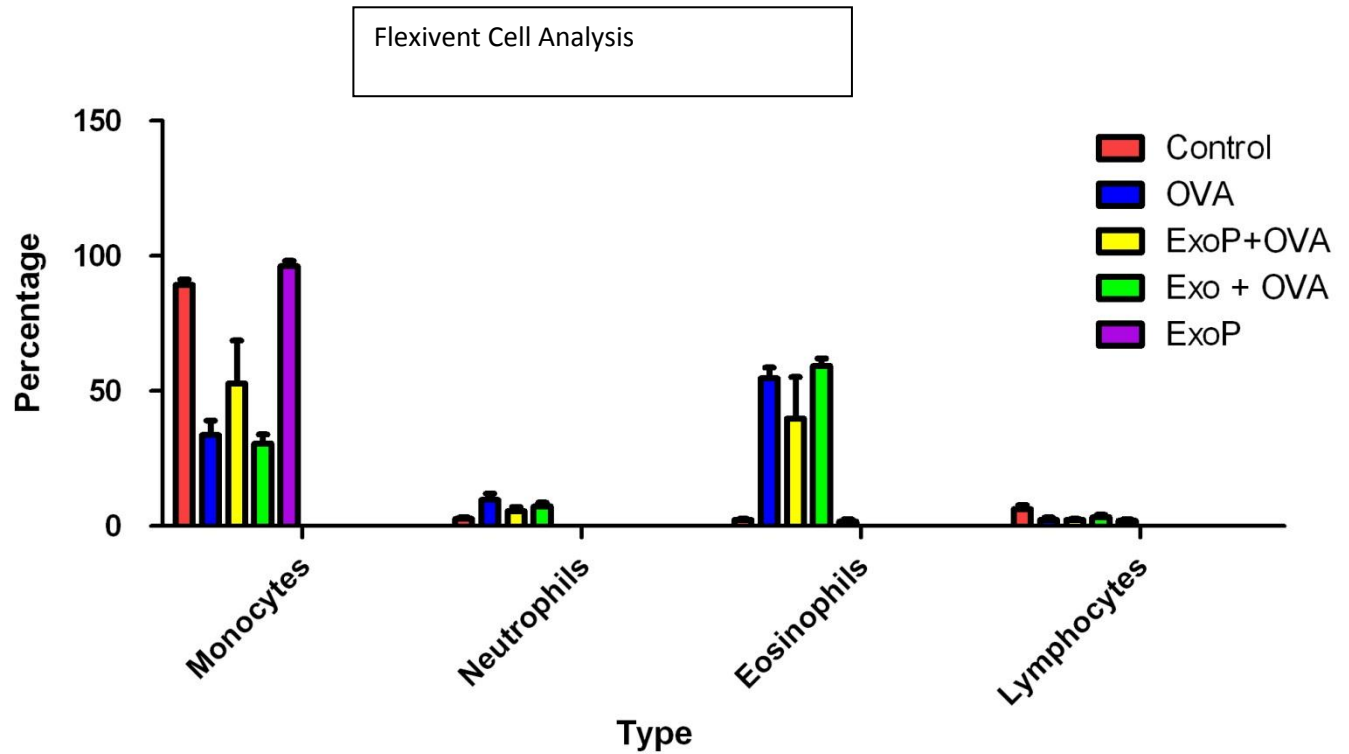
Figure 29



Mast Cell Dependent Model of Airway Hypersensitivity

Balb/c mice were sensitized by the i.p. injection of 50 µg of OVA in 100 µL of saline on days 1, 3, 5 and 9. On day 22 the mice were challenged i.n. with (i) exosomes plus 200 µg of OVA in 20 µL of PBS, (ii) exosomes carrying OVA peptide (ExoPep), (iii) ExoPep plus 200 µg of OVA, or (iv) 200 µg of OVA in 20 µL of PBS and again on day 25 and 28. After the final OVA challenge (day 29), the mice were sacrificed and BALF was collected.

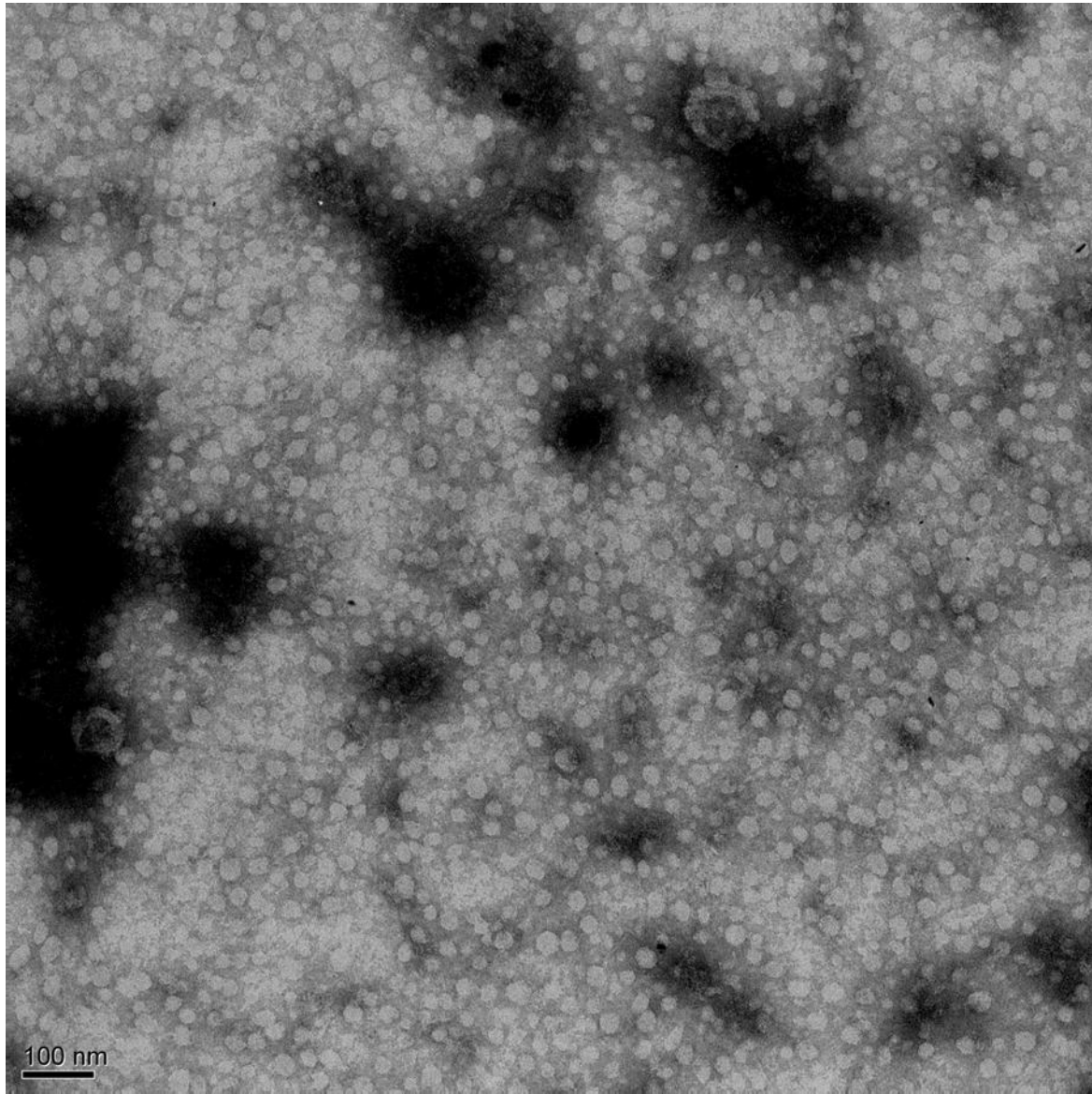
Figure 30



AHR BALF Cell Counts

After the final OVA challenge, the mice were sacrificed. BALF was collected by lavaging the lungs and then centrifuged. Pelleted cells were resuspended in 100 μ L PBS, counted, cytopun onto slides. Cells were counted by visual inspection. Percentages were determined by counting of at least 200 cells per slide.

Figure 31



For the negative staining, exosomes were placed on formvar-coated 150-mesh copper grids. The grid, formvar side to the sample, was then placed on top of the drop of sample and allowed to rest for 1 minute. Next a drop of the stain was placed on the parafilm and the grid was put on top of it for 1 minute. After 1 minute, the grid was picked up, and the excess stain was wicked off. The grid was then allowed to dry completely before scoping. Final developing solutions were tested: 1% Uranyl acetate, which gave a grainy image, and 1% Phosphotungstic acid at pH 7.0, which gave a much smoother stain around the exosomes.

VI. Conclusions

For over 100 years, it has been known that antibody aggregation increases binding to Fc receptors and results in the increase in the uptake and processing of antigen.⁵⁶ In this regard, the injection of antigen bound to an antibody leads to more than a 1000-fold increase in antigen specific antibody response.⁵⁷ Moreover, this observation true for IgE, which is dependent upon CD23 expression, as this enhancement is not present in mice deficient in CD23. It also has been shown that when mice are injected with IgE-antigen complexes also display an increase in antigen specific T cell proliferation.⁵⁸

Prior to this past year, the CD23-mediated enhancement was ascribed to B cell antigen processing. However, the finding that B cells were only required to transport the IgE immune complexes to the spleen challenged this assumption. The Heyman paper asked whether their study, which showed that CD19⁺/CD23⁺ B cells transport IgE-antigen to the spleen where the antigen is captured and presented to CD4 T cells by CD11c⁺ cells, could be explained by either pinocytosis, trogocytosis, endocytosis or exosomal transfer. The results presented in this thesis would suggest that CD23⁺ exosomes play some role, with or without the participation of CD11c⁺ cells, in antigen transfer. There is no indication, however, that exosomes solely are responsible for antigen transfer. B cells could still be at least partially involved in the antigen processing activities. Most likely, many different cells –APCs, T cells and B cells – are involved in transferring antigenic information at the immune synapse.

While there are published studies that demonstrate a role for B cell derived exosomes in the activation and proliferation of T cells, none have focused upon the potential role of CD23 as a molecular basis for this phenomenon, at least in the context of allergy and asthma. This thesis provides direct evidence that B cell-derived exosomes possess co-stimulatory molecules,

including CD80 and CD86, which act in concert with CD23 to induce T cell proliferation, at least *in vitro*. This is due to, or enhanced by, the exosomal transfer of the antigen or peptide to T cells. As also evidenced by the DO11.10 T cell proliferation studies (Figures 23-28), the activation is dependent upon the availability of IgE and the expression of CD23.

B cell exosomes can be loaded with antigen complexes and could serve as a source of recycled antigen for uptake by antigen presenting cells. However, there is little evidence that this is an actual *in vivo* process. For example, many *in vivo* studies of exosome function involve *in vitro* co-incubation (pulsing) of the exosomes with large concentrations of antigen or peptide in excess of natural conditions prior to adoptive transfer. In that connection, our lab has performed several adoptive transfer experiments (data not presented) using OVA-peptide loaded exosomes and transplanted DO11.10 T cells in an attempt to elicit OVA peptide specific T cell proliferation. Spleens taken from mice were taken and stained for DO11.10, CD4 and CD3 ϵ and analyzed by flow cytometry. There are some indications that peptide-loaded exosomes do elicit DO11.10 T cell proliferation *in vivo*.

This data is suggestive of peptide-loaded exosome induced T cell activation and proliferation, but it is not conclusive. One possibility is that the adoptive transfer of exosomes isolated from only 5 million B cells is not sufficient to cause a physiologically significant reaction. In contrast, the i.v. injection of OVA-IgE immune complexes, which would utilize the recipient's entire B cell repertoire, does induce a definitive T cell proliferative response, but the amount injected (20 μ g OVA/50 μ g IgE) is much higher than what would be present in exosomes from 5 million B cells. Another possibility is that the exosomes initially traffic to the spleen, but they remain there only momentarily and then are dispersed to other organs or tissue. Alternatively, the exosomes, by themselves, may not transfer to the spleen with any efficiency.

Future experiments, using dye labeled exosomes would be a way to test both this and as well, determined the best methodology to get dendritic cell loading of the exosomes. As mentioned above, I have tried experiments (see Figure 32) where LPS activated DC are first incubated with the exosomes and then both DC and remaining exosomes are injected, but no significant DO11.10 T cell migration into the spleen was seen (Figure 33).

Two future experiments to test these questions would be to directly inject the exosomes into mouse spleen. This has been reported to be effective for monoclonal antibody preparation.⁵⁹ In addition, increasing the exosome production by using cell lines such as M12, a murine B lymphoma cell line, would allow us to do a better dose response study. An early experiment indicated that exosomes from IL-4 and anti-CD40 stimulated M12 cells incubated with immune complexes produced a greater increase in proliferation of DO11.10 CD4⁺ T cells *in vivo* when injected into CD23KO animals than OVA alone. Additional studies would confirm or provide additional information on this data.

The ability of exosomes to stimulate both CD4 and CD8 mediated T-cell responses, as well as antitumor responses, is suggestive of an antigen-presenting role.⁶⁰ However, it is unclear how much of this function relates to the direct acquisition of antigen carrying exosomes by T cells, or the indirect processing of exosomal antigen by host DCs that later transfer the antigen to T cells. My study has significant implications for understanding a potential use for exosomes. If the dosage/delivery problems can be solved, the exosomal method of immunization would have potential for both the pathogen and tumor vaccine areas of study.

Finally, there is currently a considerable amount of interest in the micro-RNA that is associated with exosomes. Another future study could look at the mi-RNA that is bound to the B cell exosomes and determine if any of the mi-RNA types would enhance the antigen presentation

and processing activities. This has the potential of greatly enhancing the exosome activity making smaller amounts of exosome/antigen needed for immunization.

Figure 32 Adoptive Transfer Experiments

B cells, incubated with IL4 (2,000 units/mL), anti-CD40 (1 µg/mL) and IgE (50 µg), and then co-incubated with LPS-stimulated (1 µg/mL) CD11c⁺ dendritic cells can stimulate the proliferation of DO.11.10 T cells in vivo in mice.

Group 1 - B220 exosomes, incubated with IL4, anti-CD40 and IgE 50 µg, then 5 µg boost next day, 4 hours later added 20 µg OVA and stimulated CD11c⁺ cells for 4 hours.

Group 2 – Group 1 exosomes, plus additional injection of 20 µg OVA

Group 3 - B220 exosomes, incubated with IL4 and anti-CD40, but no IgE or OVA added to culture and stimulated CD11c⁺ cells for 4 hours, plus 20 µg OVA added just prior to injection.

Group 4 – Group 3, but no OVA injection

Group 5 – injection of IC (50 µg IgE/20 µg OVA)

Day 1

B220 cells + IgE/anti-CD40/IL4

Day 2

Wash cells and add 1 µg IgE boost/anti-CD40/IL4

Day 3

Isolate exosomes at 50 µL concentration

Isolate CD11c⁺ cells and stimulate with LPS (1 µg/mL) overnight

Inject DO11.10 T cells into Groups. Keep T cell sample as unstained control for Day 7 flow.

Day 4

Wash CD11c⁺ cells twice in fresh media to remove LPS. Incubate 1,250,000 CD11c⁺ cells with exosomes for 5 hours with 20 µg OVA 96 well plate. Inject into Balb/c mice.

Day 5-6

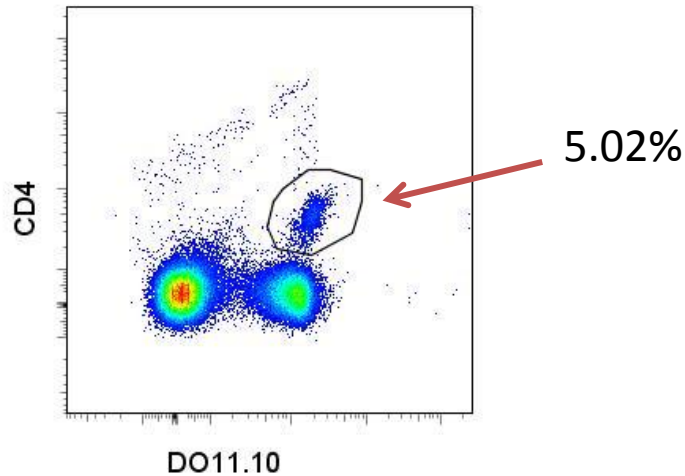
Wait

Day 7

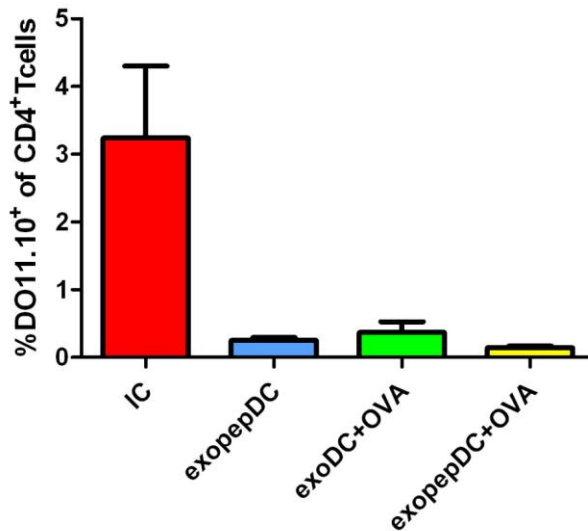
Harvest spleens and run flow for DO.11.10 cell proliferation.

Figure 33

1. Immune Complex Group –DO11.10 specific proliferation



2. All Experimental Groups –DO11.10 specific proliferation



DO11.10 Proliferation from Experimental Groups discussed in Figure 32

1. Immune Complex Group showed 5.02% DO11.10 T cell specific proliferation in spleen. The DO11.10 population is evidenced by CD4^{hi}/DO11.10^{hi} markers. Data taken from BD FACSCanto II flow cytometer and analyzed using FlowJo software.
2. Compilation of each group (n=3). The experimental groups showed little DO11.10 T cell proliferation in the spleen. This may be because (a) peptide carrying exosomes from 5 million B cells are insufficient to induce a meaningful response or (b) exosomes do not traffic to the spleen follicles.

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VITA

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